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Responses in the Guinea Pig Optic Nerve Four Hours after Stretch-Injury

By

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**A Thesis Presented for the Degree of Doctor of Philosophy in the
Institute of Biomedical and Life Sciences, University of Glasgow**

March 1998

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**In The Name of God The Compassionate,
The Merciful**

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SUMMARY

Morphological and quantitative morphometric analysis of changes 4 hours after traumatic axonal injury (TAI) in stretch-injury to guinea pig optic nerve fibres, a model of human diffuse axonal injury (DAI), demonstrated that the response of different sizes of axons/fibres with particular respect to the axonal cytoskeleton differed. The site of maximum axonal/fibre damage was found to be in the middle rather than the prechiasmatic portion of the guinea pig optic nerve. The number of axons with a diameter $< 0.50\ \mu\text{m}$ increased significantly after stretch-injury.

Neurofilament compaction occurred in smaller axons with a diameter of less than $1.50\ \mu\text{m}$. In the axons with a diameter $< 0.50\ \mu\text{m}$ compaction of neurofilaments was associated with a reduction in both axonal calibre and the g-ratio, and associated with an increase in the number of myelin lamellae. In axons with a diameter of less than $1.00\ \mu\text{m}$ neurofilament compaction was associated with a significant increase in the number of neurofilaments. The degree of neurofilament compaction decreased with increasing axonal size. In the majority of larger fibres (axonal diameter $> 2.00\ \mu\text{m}$) compaction of neurofilaments did not occur, but rather there was a significant increase in spacing between neighbouring neurofilaments, referred to as “dispersion”, that was associated with a significant loss of their number. In a small proportion of larger fibres, in which periaxonal space(s) occurred, there was, however, compaction of neurofilaments rather than an increase in their spacing. This was associated with a significant reduction in both axonal calibre and the g-ratio, but without any change in the number of myelin lamellae. Longitudinal thin sections revealed that there were local foci of compaction of neurofilaments along the length of some nerve fibres that

were separated from regions where the appearance of the fibre was close to normal. At these foci, compaction of neurofilaments was associated with the occurrence of large holes in the axolemma, separation of the axolemma from the remnants of the cytoskeleton and loss of the close relationship of the axolemma to the myelin sheath. The myelin sheath at these foci was grossly disrupted and expanded to form a “balloon-like” appearance.

The response of microtubules differed from that of neurofilaments after stretch-injury. There was an apparent increase in spacing between microtubules in fibres with an axonal diameter less than 1.50 μm and decrease in their number in fibres with axonal diameter less than 2.00 μm , however these changes were not statistically significant. There was a significant increase in spacing between microtubules in fibres with an axonal diameter greater than 1.50 μm . There was a loss in their number in both larger fibres (diameter > 2.00 μm) with dispersion of neurofilaments and those with periaxonal space(s) where compaction of neurofilaments occurred.

It is hypothesised that there is a spectrum of axonal damage. At milder levels of injury there is misalignment of the cytoskeleton, at more severe levels compaction of neurofilaments, loss of microtubules and fragmentation of the axolemma. Morphological and morphometric analysis provided novel data which demonstrated that (i) the response by neurofilaments differed in different sizes of axons/fibres, (ii) that the neurofilament response differed along the length of the same axon, and (iii) the response by microtubules to stretch-injury differed from that of neurofilaments. The present study provides the first evidence that there is indeed a spectrum of pathological changes in different sizes of axons/fibres 4 hours after traumatic axonal injury to guinea pig optic nerves.

CHAPTER ONE

INTRODUCTION

I. Morphology of Normal Myelinated Axons

I.1. General characteristics of myelinated axons in the CNS and PNS

The fact the myelin sheath is formed from compact glial lamellae that helically wind around the axon, was first observed in the chick sciatic nerve (Geren, 1954). The theory of myelinogenesis proposed by Geren (1954) was supported by Robertson (1955), working on adult chameleon myelinated peripheral nerve fibres, who suggested that a double layer membrane wound helically around the axon to form the layered myelin structure. Some time after these studies, the fine structure of myelin was elucidated in the central nervous system (CNS) where early electron microscopic studies of amphibian and rat central nervous system showed that compacted glial lamellae wind around central nervous system axons in an arrangement largely similar to the arrangement in the peripheral nervous system (PNS) (Maturana, 1960; Peters, 1960). But the periodicity of the peripheral myelin sheath has been found to be 10% larger than in CNS (Karlsson, 1966) and the Schmidt-Lanterman incisures occur much more frequently in the peripheral myelin sheath than in the central nervous system (Blakemore, 1969; Hirano *et al.*, 1969a). There are similarities and differences in the protein composition of CNS and PNS myelin. The major structural protein which comprises 50% of CNS myelin protein content is proteolipid protein (PLP). The second most abundant protein in CNS and PNS myelin is myelin basic protein (MBP) (Monuki and Lemke 1995), while in PNS the most abundant protein is P_0 (see Monuki and Lemke, 1995). Peripheral myelin protein 22 (PMP-22) is incorporated into compacted regions of PNS myelin (see Monuki and Lemke, 1995). Myelin-associated glycoprotein (MAG) comprises about 1% of myelin protein in both PNS and CNS myelin; it is present in compacted regions of myelin (Monuki and Lemke, 1995). P_0

appears to mediate intraperiod and major dense line compaction in PNS myelin, while this is accomplished in the CNS by both MBP and PLP (Monuki and Lemke, 1995).

The myelinated axon originates from the neuronal perikaryon. Unlike the dendrite, which can taper sharply, the axon has a constant diameter throughout its length even though it may extend for a considerable distance, (Peters, 1968; Friede, 1970). It is not a characteristic of axons to branch or form synaptic contacts before termination, although some axons do branch and have collaterals in the nodal region (Khattab, 1966; Lieberman *et al.*, 1972; Palay and Chan-Palay, 1974; Waxman, 1975b; Lange 1976). The occurrence of post synaptic specialisation is not common at nodes of Ranvier (Peters *et al.*, 1991), but some nodes of Ranvier form synapses where the node contains a presynaptic specialisation (Bodian and Taylor, 1963; Khattab, 1966; Sotelo and Palay, 1970; Kohno *et al.*, 1972; Pappas and Waxman, 1972; Waxman, 1972, 1974). The formation of axonal synapses occasionally occurs where there is a postsynaptic element at the initial segment or in the terminal branch (Peters *et al.*, 1991).

Nodes of Ranvier separate the segments of myelin sheath along the length of an axon similar to the beads on a string. The length of the internode is generally proportional to the axon diameter (Bodian, 1951; McDonald and Ohlrich, 1971). The longitudinal extent of adult CNS internode has been reported to increase from 100 μm to 1700 μm , as fibre diameter increases from 1 μm to 17 μm in spinal cord of cats (McDonald and Ohlrich, 1971; Gledhill and McDonald, 1977; Murray and Blakemore, 1980) and rabbits (Hess and Young, 1952) where the relationship between external fibre diameter and internodal length is essentially a linear function (Murray and Blakemore, 1980). There is a linear relationship between myelin sheath thickness and

axonal calibre in the central nervous system (Waxman and Bennett, 1972; Waxman, 1975b). The sheath thickness of a myelinated axon also increases as it extends into the peripheral nervous system (Maxwell *et al.*, 1969; Nemecek *et al.*, 1969). Axons form the bundles of parallel cell processes that comprise the white matter tracts of the central nervous system. The interfascicular oligodendroglia, which form myelin in the central nervous system, appears within these tracts as chains of small cells. Astrocytes which form junctions with the oligodendrocytes are interspaced among these cells.

The myelin sheath generally occurs in relation to the axon. However, myelin sheaths have also been documented in relation to other cell processes and perikarya (Rosenbluth, 1966; Bignami and Ralston, 1968; Hirano *et al.*, 1968; Blinzinger *et al.*, 1972; Cook, 1974; Kemali, 1974; Cooper and Beal, 1977) for example the perikarya in some peripheral ganglia (Peters *et al.*, 1991); certain dendrites found in the olfactory bulb of normal adult monkeys (Pinching, 1971) and cats (Wiley, 1973) are myelinated.

I.2. Transverse section profile of the myelin sheath

In the internodal region of central myelinated axons the cross section of the axon appears as nearly circular. The circularity of an axon is determined by the parameter \varnothing which is defined as the ratio of the axonal area to the area of the circle having the same circumference as the axon (Arbuthnott *et al.*, 1980). \varnothing has a maximum value of 1.0 for trully circular axons and declines as circularity is reduced. The multilamellar myelin sheath surrounds the axon by winding spirally around it, the outer end of the spiral being formed by an outer cytoplasmic loop that is surrounded by oligodendroglial plasma unit membrane. The fusion of apposed inner leaflets of the plasma unit membrane form the major dense line of the myelin lamella. The minor dense or

intraperiod line, which may occasionally be observed as two lines, is formed by the juxtaposition of the outer leaflets (Hirano *et al.*, 1966). Adjacent to the axon at the inner end of the spiral, the major dense line separates at the inner mesaxon and surrounds an inner cytoplasmic loop. The inner and outer loops usually are located in the same quadrant in about 75% of myelin sheaths (Peters, 1964).

Similar size and configuration are normally seen in the outer and inner cytoplasmic loops. Except for a few microtubules, fine granules and a rare vesicle, the loops do not usually contain formed organelles. They may contain occasional filaments (Hirano and Zimmerman, 1971a), especially in certain pathological conditions (Cavanagh *et al.*, 1971). The outer lamella is connected to the outer loop by a linear tight junction (Mugnaini and Schnapp, 1974). Disruption of this tight junction may cause separation of the lamellae allowing penetration by extracellular fluid (Lampert, 1968; Hirano *et al.*, 1971; Bornstein and Raine, 1976; Hirano, 1972, 1992). Alternatively, under some pathological conditions, for example triethyl tin intoxication, the tight junction does not disrupt and remains intact, but there are focal separations of the lamellae without any recognisable extracellular content (Aleu *et al.*, 1963; Hirano *et al.*, 1969b; Tabira *et al.*, 1978; Greco *et al.*, 1980; Hirano, 1969, 1972, 1992).

A narrow extracellular space, termed the periaxonal space, separates the axon from the inner glial loop and innermost myelin lamella. The extent of the periaxonal space is remarkably resistant to change despite remarkable pathological alterations in nerve fibres. For example in hind leg paralysis in the Syrian hamster there is excessive swelling of the axon, enlargement of inner loop of the Schwann cell and abnormally small axons (Homburger and Bajusz, 1970). Despite this the width of the periaxonal space is essentially maintained as normal. However, sometimes when the axon is

unusually small, the periaxonal space is enlarged to a great extent and may have a variable electron-dense material (Hirano and Dembitzer, 1981).

I.3. The relations between axon and myelin sheath

Most axons in the mammalian central nervous system are myelinated, while most in peripheral nerves are unmyelinated. Studies on the feline spinal cord (Hildebrand and Skoglund, 1971), pyramidal tract of rabbits (Franson and Hildebrand, 1975), callosal and spinal fibres of cats (Remahl and Hildebrand, 1982) and rat optic nerve fibres (Hildebrand and Waxman, 1984) have demonstrated that the vast majority of myelinated CNS axons are very thin and have fibres with an axonal diameter ranging from 0.2 μm to 1.5 μm . In CNS tracts unmyelinated axons have diameters up to 0.8 μm . Since these axons lack glial ensheathments, they are directly exposed to the extracellular space. Generally, there is a relationship between axon calibre and myelin sheath thickness in the central nervous system (Friedrich and Mugnaini, 1983; Waxman and Sims, 1984). Almost all axons with diameter above 0.2 μm in the central nervous system are myelinated (Bunge *et al.*, 1967; Samorajski and Friede, 1968; Hirano and Zimmerman, 1971b; Matthews and Duncan, 1971; Waxman and Bennett, 1972; Waxman, 1975a, b; Waxman and Swadlow, 1976), where often some axons with a diameter of about 0.2 μm had about 10 myelin lamellae in their myelin sheath (Hirano and Zimmerman, 1971b). More recently, however, studies on the dorsal funiculus of the rat spinal cord (Matthews and Duncan, 1971), postnatal growth in the pyramidal tract of the rabbit (Franson and Hildebrand, 1975), in feline developing white matter (Remahl and Hildebrand, 1982) and in postnatal development of the rat

optic nerve (Hildebrand and Waxman, 1984) have demonstrated considerable overlap in the size spectra of myelinated and unmyelinated axons.

Duncan (1934) and Young (1945) proposed that the diameter of the axon may be an important factor in determining the extent of myelination of a nerve fibre. They suggested that the size of axon may be the main factor to determine whether or not a nerve fibre has a myelin sheath, since all fibres thicker than a critical diameter are myelinated. This critical diameter was determined to be between 1.0 and 2.0 μm in the PNS. In the PNS the axonal diameter of the thinnest myelinated fibres was 0.8 μm for embedded tissue and 1.1 μm for fresh tissue (Friede and Samorajski, 1967). In the PNS it has been found that the number of myelin lamellae has a linear relationship to axon circumference in both the vagus and sciatic nerves of mice (Friede and Samorajski, 1967). It has also been documented that in fresh, unfixed myelinated fibres of the same nerves and animals, there was one myelin lamella for every 0.24 μm increase in axon circumference above a threshold value of 2.32 μm (Friede and Samorajski, 1967). Later investigations extended these ideas, and the relationship is generally considered to be rectilinear (reviewed by Friede 1972; Bischoff and Thomas, 1975). Other studies, however, have suggested that the relationship is only linear below an axon diameter of 4-5 μm (Boyd and Kalu, 1973; Sima, 1974; Berthold and Carlstedt, 1977). Hildebrand and Hahn (1978) found that the number of myelin lamellae is related to axon diameter according to a curvilinear function and that the slope of the line for this function varies between species, with the number of myelin lamellae in ventrolateral funiculus white matter fibres of the spinal cord in cat, rabbit, guinea pig, rat and perch demonstrating a regular increase with axon size up to about 4-5 μm corresponding to 60-70 lamellae. However, above this size they have shown

that the relation between the number of myelin lamellae and axon size is less strict with the number of myelin lamellae rising more slowly as the diameter of the axon increases. In axons with a diameter above 10 μm in the ventrolateral funiculus white matter the mean number of myelin lamellae were found to vary irregularly between 100 and 140. In all of these species the number of the myelin lamellae showed a pronounced variability in the largest fibres (Hildebrand and Hahn, 1978).

Other properties of myelin sheaths in the CNS also vary with diameter of the axon. Ultrastructural analysis has established that while the lamellar spacing of native CNS myelin is about 16 nm, the myelin period is some 8 nm in the thick sheaths of large CNS axons, and about 12 nm in the thin sheaths of small CNS axons (Karlsson, 1966; Hildebrand, 1971; Hildebrand, 1972; Hildebrand and Müller, 1974). These differences arise during preparative dehydration and embedding for TEM (Hildebrand and Müller, 1974). It has recently been suggested that these differences reflect different effects of lipid solvents on thick and thin sheaths during dehydration and embedding (Hildebrand *et al.*, 1992). Indeed, it has been established that lipid and protein composition of CNS myelin sheaths may differ between large and small axons (Norton and Cammer, 1984). This may correlate with the fact that large and small CNS axons are associated with different types of oligodendrocytes (Hildebrand *et al.*, 1992).

I.4. The relationship between axon diameter and fibre diameter

The ratio between axon diameter and total fibre size is a parameter of prime importance for the functional properties of myelinated nerve fibres. This ratio (g-ratio), which reflects the longitudinal resistance of the axon and the impedance of the myelin sheath, has been used in theoretical calculations concerning the axonal

conduction velocity (Rushton, 1951; Goldman and Albus, 1968; Deutsch, 1969; Smith and Koles, 1970). Conduction velocity varies linearly with diameter in myelinated fibres, and is maximal when the g-ratio has a value close to 0.60. It has been proposed that the optimal g-ratio for attaining effective internodal spread of current is between 0.60 and 0.80, with the range being due to some inter-species variation (Goldman and Albus, 1968; Jack *et al.*, 1975; Williams and Chalupa, 1983). In fact, in most CNS myelinated fibres, irrespective of diameter, the myelin thickness is matched to axon diameter in such a way that the g-ratio falls close to the optimal value of 0.6 (Waxman and Bennett, 1972). There is a consensus among most investigators in support of constant g-ratio in central nervous system fibres (Friede and Samorajski, 1967; Samorajski and Friede, 1968; Waxman and Bennett, 1972; Fraher, 1972, 1976; Waxman and Swadlow, 1976; Friede and Beuche, 1985). But, while Ogden and Miller (1966) provide data that the g-ratio increases with fibre size; Hildebrand and Hahn (1978) estimated the g-ratio of small fixed and embedded fibres to be about 0.70 in all species, although there is variation in the g-ratio in the largest nerve fibres of mammals. The values of the g-ratio in the smallest and largest fibres obtained by Hildebrand and Hahn (1978) in a variety of vertebrates ranged from:- cat 0.73-0.85, rabbit 0.69-0.86, guinea pig 0.71-0.84, rat 0.65-0.82 and mouse 0.73-0.84 in mammals and in perch 0.69-0.90 and frog 0.73-0.94. Thus it has been demonstrated that the number of myelin lamellae increases with axon diameter up to a certain axonal size (4-5 μm in diameter). Above this size there is a lesser increase in the number of myelin lamellae with increasing axonal diameter (see pages 7-8) (Hildebrand and Hahn, 1978). Thus the g-ratio differs between smaller and larger fibres.

I.5. Cross sectional morphology of the axon

In comparison with the soma, an axon contains fewer cytoplasmic organelles, because it does not have Golgi apparatus and rough endoplasmic reticulum. Elements of smooth endoplasmic reticulum (axoplasmic reticulum) are seen as circular or cistern-like figures in cross sections of central myelinated axons. However, high-voltage transmission electron microscopy of thin sections has shown that the axoplasmic reticulum forms an elaborate, reticulated, tubular network extending from the axon hillock to the nerve ending (Droz *et al.*, 1975). It has been suggested that this system to take part in fast bi-directional axonal transport (Droz, 1975).

Mitochondria are generally oblong and oriented parallel to the long axis of the axon, and occasionally possess branches. The arrangement of the mitochondrial shelf-like cristae is generally parallel to the long axis of the axon. The cristae are damaged in some pathological conditions, such as anoxia (Waxman *et al.*, 1992) or non-disruptive axonal injury (Pettus *et al.*, 1994; Maxwell *et al.*, 1995, 1997b).

I.5.a. The cytoskeleton

The axoplasm is specialised in a way to maintain its tube like-shape and considerable length. The axoplasm is surrounded by 8 nm triple-layered thick membrane or axolemma and appears morphologically similar to the plasma membrane of the neuronal soma, but with some structural specialisations especially at the node of Ranvier and paranodal regions. In unmyelinated axons various membrane proteins appear to be uniformly distributed in the axolemma, while in myelinated axons there is a tendency for proteins to concentrate in the nodal and paranodal regions. The axolemma at the node and around it is specialised to have two apparent types of

axoglial junctions (Rosenbluth, 1988). At the node a junction is formed similar to a desmosome, with microvilli of Schwann cells in PNS and astrocyte processes in CNS (Waxman, 1986). The extracellular face of nodal axolemma shows an unusual marked distribution of 7-10 nm membrane particles which are highly concentrated at the node (Chiu, 1980; Waxman and Ritchie, 1985) and have a low concentration at paranode (Chiu and Schwarz, 1987). It has been proposed that these particles present voltage-gated sodium channels at E-face (Rosenbluth, 1976). At the paranode there are 26 nm dimeric particles in the axolemma and 16 and 7.5 nm particles in the related paranodal glial membranes (Maxwell *et al.*, 1988). These particles are hardly observed at paranodal axoglial junctional strips, but most of them are seen between the strips and sometimes in greater number in more widened strips, especially in that part of paranode that is farthest from the node (Rosenbluth, 1976; Tao-Cheng and Rosenbluth, 1980).

It has been revealed that the cytoskeleton is the most conspicuous component of the axoplasmic organelles (Burgoyne, 1991). It consists of neurofilaments, and microtubules and the microtrabecular matrix, including the actin microfilaments (Ellisman and Porter, 1980; Hirokawa, 1991). The growth pattern, the size, the shape of the axon, and the stability of the axolemma is determined by the cytoskeleton. It contains the machinery necessary for the interaction with the anterograde transport “motor” (kinesin) and retrograde “motor” (dynein) (Sheetz and Martenson, 1991).

I.5.ai. Neurofilaments

Neurofilaments are conspicuous organelles of the axon and under high magnification (e.g., X 100,000-400,000) appear as hollow cylindrical filaments or

tube-like structures 8-11 nm in diameter having a lucent core about 30 Å (3 nm) and a thick wall (Peters and Vaughn, 1967; Wuerker, 1970; Wuerker and Kirkpatrick, 1972) and connected together by 4-6 nm thick crossbridges or side-arms (Terry and Pena, 1965; Hirano, 1970; Wuerker and Kirkpatrick, 1972; Ellisman and Porter, 1980; Hirokawa 1982). It has been shown that isolated neurofilaments appeared as cylindrical structures formed from 2-2.5 nm protofilaments (Schlaepfer, 1977; Krishnan *et al.*, 1979). They are aligned parallel to the long axis of the axon (Wuerker, 1970; Wuerker and Kirkpatrick, 1972). Their density is about 150 to 300 neurofilaments per μm^2 of cross-section of the axoplasm, and they are considered to be the major determinant of the axon size (Hoffman *et al.*, 1985a; Griffin *et al.*, 1988; Hoffman and Griffin, 1993). Studies on rat spinal cord has revealed that the density of neurofilaments varies in different tracts. Szaro and colleagues (1990) demonstrated that the density of neurofilaments in the myelinated cuneate fibres (237.7 per μm^2) was two and half times greater than the density in corticospinal tract fibres (93.1 per μm^2). They also demonstrated that the density of neurofilaments in axons with a diameter of above 2.0 μm (263.5 per μm^2) was greater than the density in smaller axons (228.7 per μm^2). More recently, Maxwell and Graham (1997) demonstrated that the highest number and density/ μm^2 or 0.0206 μm^2 of neurofilaments occurred in the internode of larger axons in guinea pig optic nerve. Unlike neurofilaments in the perikaryon and dendrites, they are usually highly phosphorylated in the axon (Sternberger and Sternberger, 1983; Matsumoto *et al.*, 1989; Mizusawa *et al.*, 1989). Three separate protein elements which are collectively called neurofilament triplet proteins (Hoffman and Lasek, 1975), are polymerised to form neurofilaments. Each of them is synthesised from a different mRNA and separate gene (Myers *et al.*, 1987; Lees *et al.*, 1988;

Steinert and Roop, 1988). The neurofilament subunits have a highly conserved central α -helical 'rod' domain, an amino-terminal head domain, and carboxy-terminal 'tail' domain. The three polymeric proteins have molecular weights of 68, 150, and 200 kD (NF-L, NF-M, and NF-H respectively) as estimated by gel electrophoresis (Dautingy *et al.*, 1988), share a central rod domain, but their carboxy-terminal regions are different. The NF-M and NF-H are cross-linking proteins assembled with NF-L which is found predominately in the neurofilament core (Posmantur *et al.*, 1994). Examination of a single *in vitro* neurofilament, either isolated or reassembled, shows that it is made from a central filamentous portion 10-12 nm thick called core filament, and projections that are formed from thinner neurofilamentous portion 4-5 nm thick, extend perpendicularly, regularly and bilaterally from the core filament (Hisanaga and Hirokawa, 1988; Gotow *et al.*, 1992). The α -Helical rod domains of all three subunit proteins are considered to build the core filament (310 amino acids), while carboxy-terminal tail domain of two larger subunits NF-M and NF-H form the projections (400-600 amino acids) (Geisler *et al.*, 1983, 1985; Lewis and Cowan, 1985; Liem *et al.*, 1985; Hisanaga and Hirokawa, 1988; Mulligan *et al.*, 1991; Gotow *et al.*, 1992; Nakagawa *et al.*, 1995). The cross-bridges between neurofilaments are formed when the distance between the core of two neurofilaments is short enough for their apposed projections to interact with each other (Leterrier and Eyer, 1987; Gotow *et al.*, 1992). The cross-bridges, 4-5 nm in width, are specific structure to neurofilaments. A minimum space is maintained between individual neurofilaments by cross-bridges or radially projecting side-arms (Pant *et al.*, 1978; Leterrier *et al.*, 1982; Liem and Hutchinson, 1982; Sharpe *et al.*, 1982; Julien and Mushynski, 1983; Hirokawa *et al.*, 1984; Nakagawa *et al.*, 1995).

It was in 1982 when Lazarides first suggested that phosphorylation may regulate the deposition and function of intermediate filaments. Soon thereafter, it was found that neurofilament proteins, especially the high molecular weight subunits NF-M and NF-H, contain a large number of phosphate groups on their carboxyl-terminal tail regions (Julien and Mushynski, 1983; Carden *et al.*, 1985). The major phosphorylation site of NF-M and NF-H subunits is the carboxyl-terminal which contains a highly repeating lysine-serine-proline (KSP) sequence (Geisler *et al.*, 1987; Lee *et al.*, 1988). In NF-H the carboxy-terminal region contains over 40 lysine-serine-proline repeats (Julien and Mushynski, 1982,1983), which provide potential phosphorylation sites (Black and Lee, 1988; Lee *et al.*, 1987, 1988; Julein *et al.*, 1988). It has been revealed in an examination of the predicted amino acid sequences for rat NF-H (Lieberburg *et al.*, 1989) and NF-M (Napolitano *et al.*, 1987) that one class of potential phosphorylation site (the serine in the sequence of lys-ser-pro) is repeated 5 times in NF-M and 44 times in NF-H. NF-M and NF-H are the most exclusively phosphorylated proteins in the brain (*in vivo*) containing 15 to 50 mol of phosphate respectively (Julien and Mushynski, 1983; Carden *et al.*, 1985).

The protein kinases that are involved in phosphorylation of multiple phosphorylation sites of amino-terminal head domains of NF-L and NF-M are distinct from those that phosphorylate carboxyl-terminal domains (Sihag and Nixon, 1989, 1990, 1991). Kinase cdk2 is likely to be responsible for phosphorylation of axonal NF-H and NF-M (Lew *et al.*, 1992). It has been suggested that neurofilament polymerisation may be regulated by phosphorylation/dephosphorylation of specific amino terminal sites on NF-L and NF-M where adding phosphate to the head domain of NF-L by protein kinase A, *in vitro*, disassembles NF-L filaments (Hisanaga *et al.*,

1990; Nakamura *et al.*, 1990). Control of assembly/disassembly of neurofilaments by phosphorylation/dephosphorylation of amino-terminal head domains varies in different compartments of even the same neuron (Langley *et al.*, 1988; Angelides *et al.*, 1989). The relative strength of neurofilament interactions with transport carrier and with stationary axonal structures is regulated by phosphorylation of carboxy-terminal, and therefore it controls the rate of movement and the time that neurofilaments are present within an axon (Lewis and Nixon, 1988; Nixon and Sihag, 1991).

Glicksman and colleagues (1987) hypothesised that upon the phosphorylation of NF-H, the projections are stiffened and extend perpendicular to the core filament to form regular cross-bridges. This would be difficult if NF-H were not phosphorylated. Further, de Waegh and colleagues (1992) proposed that the space between core filaments is maintained when phosphorylated neurofilament side-arms, highly charged with phosphates, are repelled from core filament surfaces and from opposed projections, but that dephosphorylated projections with little charge allow core filaments to close the space. This hypothesis, however, was not supported by the findings of Hisanaga and Hirokawa (1989) in an *in vitro* experiment which demonstrated that after removal of almost 90% of phosphates from the carboxy-terminal on the NF-H subunit, there were no significant difference in relations of projections or cross-bridges between phosphorylated and dephosphorylated neurofilaments. In reviewing the latter finding Nixon and Sihag (1991) questioned this early study and proposed that it should be known whether the amount of phosphate that remains on the carboxy-terminal tail domain would be enough to maintain the configuration of the side-arm after it is fully extended. Nixon and colleagues (1994) have demonstrated in retinal ganglion cell axons of mice that upon the transportation

of neurofilaments, NF-M and NF-H subunits start to reach a mature state of phosphorylation within the very proximal region along the optic nerve axons beginning 150 μm from the retinal excavation, and this increase in phosphorylation of the carboxy-terminal tail domain is correlated with an increased average and minimum obligatory spacing between neurofilaments.

It is now widely accepted in different animal species that neurofilament proteins are significantly more phosphorylated in axons than in cell bodies and dendrites (Sternberger and Sternberger, 1983; Carden *et al.*, 1987; Cohen, Pant, House and Gainer, 1987; Lee *et al.*, 1987; Szaro and Gainer, 1988). Investigations using ^{32}P -incorporation into neurofilament protein have demonstrated that neurofilament proteins are largely phosphorylated in axons (Oblinger, 1987; Black and Lee, 1988) and phosphorylation continues as they are transported through the axon (Nixon and Lewis, 1986; Nixon *et al.*, 1982, 1987). Highly phosphorylated neurofilaments in myelinated axons do not appear to be phosphorylated regularly along the length of the axon. Antibodies to phosphorylated epitopes showed 60% and 40% reduction in phosphorylation of NF-H and NF-M respectively in the node of Ranvier compared to the internode of rat sciatic nerves, while there was no change in NF-L phosphorylation at the node of Ranvier (Mata *et al.*, 1992). Gotow and Tanaka (1994) demonstrated that projections or cross-bridges in NF-H with other organelles are not phosphorylated when neurofilaments are dispersed singly, but when neurofilaments form bundles, the cross-bridges of NF-H between the core filament of neurofilaments are phosphorylated. This probably explains why in axons in which neurofilaments are abundant, NF-H is heavily phosphorylated, whereas it does not occur in dendrites and perikarya, where neurofilaments are sparse and do not form bundles (Gotow and

Tanaka, 1994). Gotow and Tanaka (1994) provided the following explanations why NF-H is not phosphorylated when neurofilaments are not in bundles and occur singly in dendrites. Since microtubules are the more abundant of cytoskeletal components in dendrites, it is possible that phosphorylated NF-H is not suitable for interaction between neurofilaments and microtubules and microtubule-associated protein 1A and 2 in dendrites (see Gotow and Tanaka, 1994). The binding of neurofilaments and microtubules through NF-H is promoted when neurofilament proteins are dephosphorylated, or inhibited when NF-H is phosphorylated. Thus dephosphorylation is probably required for interaction between neurofilaments and microtubules (see Gotow and Tanaka, 1994). On the other hand, microtubules in the axon are likely to be separated from and be less abundant than neurofilaments, and cross-bridges are seen between neurofilaments and microtubules in the periphery of these groups (see Gotow and Tanaka, 1994). Microtubule-associated protein 2 is considered to be a possible component of neurofilament-microtubule cross-bridges in dendrites, but not in the axonal compartment, where microtubule-associated 1A, B or tau also is considered to be a possible constituent of these cross-bridges (Gotow and Tanaka, 1994). Depending on the degree of phosphorylation all three neurofilament subunits exist in many different isoforms (Nixon and Sihag, 1991). It may be hypothesised that neurofilament subunits exist as mobile monomers or oligomers when they are hypophosphorylated and that the polymers of such units are more labile.

Axonal components are transported by slow and fast axonal transport. The velocity of fast axonal transport is several microns per second, while the speed of slow axonal transport is a few microns per minute. The direction of anterograde transport is from the cell body to the axon terminal and retrograde transport is in the opposite direction

and is somewhat slower (Grafstein and Forman, 1980). Proteins that comprise membranous elements and enzymes that are related to neurotransmission are carried by fast axonal components (Grafstein and Forman, 1980; Lasek *et al.*, 1984). Slow transport of axonal components (SC) is divided into two subcomponents, (i) the slower subcomponent (SCa) and (ii) the faster (SCb). It is believed that the microtubule-neurofilament network is carried by SCa (Black and Lasek, 1980; Mori and Kurokawa, 1980; Tytell *et al.*, 1984). SCb conveys the microfilament network together with soluble proteins in the cytoplasm (Black and Lasek, 1980; Brady and Lasek 1981). Other proteins such as clathrin (Gower and Tytell, 1987; Paggi *et al.*, 1989), calpain (Karlsson *et al.*, 1992), actin (Bray and Mills, 1991), heat shock proteins (Tytell and Barbe, 1987) and calmodulin (Brady *et al.*, 1981; Spencer and Willard, 1992) are also carried by SCb. Fodrin (Levine and Willard, 1981; Lasek *et al.*, 1984) and the neuronal form of spectrin (Lazarides and Nelson, 1983; Mangeat and Burridge, 1984) are conveyed by both SCa and SCb. It has been demonstrated that in rat retinal ganglionic cells the peak of tubulin advanced together with the peak of actin and fodrin at a rate of 0.36 mm per day, while the neurofilament triplet peak advanced more slowly (McQuarrie *et al.*, 1986). The rate of neurofilament protein transport in mouse retinal ganglionic cells was determined to be 0.5-0.7 mm per day (Nixon and Logvinenko, 1986) and in normal sciatic nerve 1.38 mm per day (deWaegh and Brady, 1990), and in the peripheral nervous system averages between 0.70 and 2.0 mm per day (Hoffman and Lasek, 1975; McQuarrie *et al.*, 1986). Axoplasmic transport in the guinea pig optic nerve axon has been reported to be 0.25 mm per day (Black and Lasek, 1980). At least four different phosphorylated variants for each of NF-H and NF-M have been described in relation to neurofilament transport in retinal ganglion

cells of the mouse (Nixon *et al.*, 1982; Lewis and Nixon, 1988). Of these variants, the one that is most heavily phosphorylated leaves the transport carrier and remains in the axoplasmic lattice for up to several months, whereas neurofilaments that are phosphorylated to a lesser level or degree remain in the axon for only a short period (of the order of days) and move along the axon by slow axonal transport (Lewis and Nixon, 1988; Nixon and Sihag, 1991).

Earlier work suggested that neurofilaments form self-assembling insoluble filamentous organelles, leaving virtually no monomeric neurofilament proteins (Black and Lasek, 1980; Morris and Lasek, 1984). Newly synthesised neurofilaments enter the axon and move anterogradely within the SCa component of axonal transport (Hoffman and Lasek, 1975) via the process of somatofugal translocation of the assembled axonal cytoskeleton (Weiss and Hiscoe, 1948; Black and Lasek, 1980; Lasek *et al.*, 1992). Neurofilaments undergo no assembly-disassembly in their passage along the axon (Morris and Lasek, 1984). When the neurofilaments reach the nerve terminal, they undergo rapid proteolysis through the activity of calcium-sensitive neutral proteases (calpains) (Lasek and Hoffman, 1976; Roots, 1983).

However, more recent work questions these above hypotheses where at least some axonal neurofilament proteins remain as monomers or in non-organelle form (Angelides *et al.*, 1989; Okabe *et al.*, 1993). It is now thought that there is a capacity for local assembly of monomers into filaments within the axon (Okabe *et al.*, 1993); and that the assembly of neurofilament subunit peptides into neurofilaments may be affected/influenced by phosphorylation (Lewis and Nixon 1988; Gonda *et al.*, 1990). Also, a stationary component may exist in the neurofilamentous cytoskeleton (Nixon and Logvinenko, 1986; Hollenbeck, 1989; Watson *et al.*, 1989; Hoffman *et al.*, 1992).

Further, it has been shown that movement of cytoskeletal protein is not necessarily unidirectional (anterograde) since retrograde movement of cytoskeletal proteins has been documented in transected sciatic nerves from C57BL/6/Ola and C57BL/Ola mice (Glass and Griffin, 1991, 1994; Watson *et al.*, 1993). Ultrastructural immunocytochemical analysis of regional heterogeneity of phosphorylation along the length of normal myelinated nerve fibres has also revealed that unmyelinated regions of these fibres, including the stem process of primary sensory neurons (Hsieh *et al.*, 1994), and nodes of Ranvier (Mata *et al.*, 1992; Hsieh *et al.*, 1994), contain a much lower proportion of phosphorylated neurofilament epitopes than the myelinated regions of the same axons. Mata *et al.*, (1992) therefore suggested that post-translational modification of neurofilaments takes place at paranodes and at nodes of Ranvier as the neurofilament trabecular network moves along an axon.

Further evidence to support the hypothesis that local modulation of the neurofilamentous cytoskeleton may occur locally in different parts of an axon has been obtained from the Trembler mouse where both the level of phosphorylation of neurofilaments and their mean spacing are reduced in segments of Trembler nerves grafted into normal sciatic nerves (de Waegh *et al.*, 1992). These local effects of Trembler Schwann cells on the cytoskeleton of normal nerve axons therefore suggest that factors extrinsic to the axon can regulate neurofilament phosphorylation (de Waegh *et al.*, 1992).

I.5.iii. Microtubules

Microtubules are another component of the axonal cytoskeleton similar to microtubules in other cells (Soifer, 1975). Like neurofilaments, their arrangement is

parallel to the long axis of the axon. They are unbranched, and vary from a few to more than 1000 μm in length (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981). They are about 24 nm thick, consisting a clear lumen surrounded by a 5 nm wall; often the lumen contains a 5 nm central density (Peters and Vaughn, 1967; Hirano, 1970). There are 5-nm granules in the wall which are arranged in a helical pattern, and each helix is formed by 13 granules (Frisch, 1969; Hirano, 1970; Tilney *et al.*, 1975). They have fine side-arms (Wuerker and Kirkpatrick, 1972; Ellisman and Porter, 1980; Hirokawa, 1982). They are either arranged singly or grouped together as bundles (Lewis *et al.*, 1989) and are extended longitudinally along the axon. Nakazawa and Ishikawa (1995) observed that fasciculated microtubules occurred at nodes of Ranvier in the proximal portion of the axons of motor and sensory spinal roots in rat. No fasciculation was found in the internodal portion of either myelinated axons or in nonmyelinated axons. In the fascicles microtubules were cross-linked by filamentous strands with a length of 12-16 nm and centre-to-centre distance between them was 36-40 nm (Nakazawa and Ishikawa, 1995). Nakazawa and Ishikawa (1995) have demonstrated that 45-48% of axonal microtubules were fasciculated at the node Ranvier of sensory axons at the level of dorsal root ganglion and that this proportion decreased in sections distal to the ganglion. In motor axons of ventral rootlets however, microtubule-fascicles involved approximately 24% of the total number of microtubules, and this was reduced distally to only 2%. There was no recognisable fasciculation of microtubules at the nodes in the distal portion of myelinated axons such as in the sciatic and saphenous nerves. Further, the packing density of microtubules varies both along a single axon and also between afferent and efferent

axons (Zenker and Hohberg, 1973; Zenker *et al.*, 1975; Pannese *et al.*, 1984; Saitua and Alvarez, 1989; Reles and Friede, 1991; Maxwell and Graham, 1997).

Microtubules and their monomeric subunits occur in a dynamic equilibrium within an axon such that about one half of the total content is in the form of polymerised microtubules and the other half is an intra-axonal pool of monomeric units in the axoplasm (Bryan, 1976). Various conditions such as the presence of taxol (Masurovsky *et al.*, 1983), colchicine or vinca alkaloids (Hirano and Zimmerman, 1970; Wiesenberg, 1972) or the concentration of calcium ions, and temperature (Rodriguez-Echandia and Piezzi, 1968; Wiesenberg, 1972; Gaskin *et al.*, 1975; Olmsted and Borisy, 1975; Nishida and Sakai, 1977; Job *et al.*, 1981; Baas *et al.*, 1994) can shift this equilibrium to either the monomeric or the polymeric state. The proximal end of a microtubule is called the minus-end (close to the cell body) and the opposite end is referred to as the plus-end (Heidemann *et al.*, 1981). Tubulin monomers and oligomers are assembled to form microtubules at the plus-end and disassembled at the minus-end. Fast axoplasmic transport of membranous organelles occurs along the tracts which are formed by microtubules (Weiss *et al.*, 1987); anterograde transport occurs toward the plus-end and retrograde toward the minus-end. Microtubules are sensitive structures. It has been demonstrated that in cultured sympathetic neurons roughly half of the microtubule mass is depolymerised in the presence of 2 µg/ml of nocodazole (Baas and Black, 1990; Baas *et al.*, 1991, 1993).

Microtubules are widely recognised as important in the transport of membranous, axoplasmic organelles (Hirokawa and Yorifugi, 1986; Raine *et al.*, 1987). Early work suggested that intra-axonal transport of mitochondria occurred by association with microtubules (Hirano and Zimmerman, 1971b; Raine *et al.*, 1971; Leopold *et al.*,

1992). Later work has shown that synaptic and coated vesicles and mitochondria are carried by fast axonal transport through anterograde movement, that is from the minus-end to the plus-end of microtubules (from cell body to the axonal terminal) and is mediated through a protein called kinesin (Leopold *et al.*, 1992). Kinesin has a heavy chain epitope at the amino-terminal end with two globular heads. The light chains form its tail (Hirkawa *et al.*, 1989; Scholey *et al.*, 1989). It is presumed that the function of the light chain is to provide binding sites for organelles transportation, and the head domain is the binding site(s) for microtubules and adenosine triphosphate (ATP) (Skoufias and Scholey, 1993). The kinesin unit can carry an organelle for many microns along the length of a microtubule (Skoufias and Scholey, 1993). Retrograde movement is mediated by dynein, another microtubule associated ATPase protein similar to motor kinesin (Vallee and Bloom, 1991).

I.5.b. Cross linkages between cytoskeleton and axoplasmic elements

Preparatory procedures such as quick freeze together with deep etching and rotatory shading, using high voltage electron microscopy for examination of unfixed tissue, or combined tannic acid and glutaraldehyde fixation have provided further information about the structure of axoplasm (Ellisman and Porter, 1980; Berthold, 1982; McDonald, 1984; Tsukita *et al.*, 1982, 1986). The cross-connection structures are the most abundant and discernible of these features. In myelinated axons of frog spinal nerves, three main type of cross-connections with thickness ranging between 4 and 6 nm have been identified (Hirokawa, 1982). Type (I) Neurofilament-associated-cross-linkers connect neurofilaments to neurofilaments and neurofilaments to microtubules and to membranous organelles, type (II) microtubule-associated-cross-

bridges connect microtubules to membranous organelles and microtubules to microtubules, and type (III) long subaxolemmal cross links connect the actin like filamentous network of the axolemma with adjacent neurofilaments and microtubules (Hirokawa, 1982). These all form a dense lattice throughout the axoplasm (Hirokawa, 1982; Schnapp and Reese, 1982; Hirokawa *et al.*, 1985; Meller, 1987). The membranous axoplasmic organelles are suspended and cross-linked to this neurofilament-microtubule lattice by cross-connections. Type (I) cross linkers are 20 to 50 nm long (Hirokawa, 1982; Hirokawa *et al.*, 1984), type (II) cross-bridges are less than 20 nm long (Hirokawa, 1982; Langford *et al.*, 1987) and type (III) are 50 to 150 nm long (Leivine and Willard, 1981; Hirokawa, 1982).

Similar findings have been revealed by Ichimura and Ellisman (1991) at the node and paranodal portions from rat spinal cord dorsal columns and sciatic nerve fibres. Subaxolemmal filaments referred as nodal membrane-cytoskeletal linkers have been shown to be involved in membrane-cytoskeletal linkage to nodal axoplasmic components. They found similar filaments at paranodal regions and called them paranodal membrane-cytoskeletal linkers. They also found three types of cytoskeleton-membrane linkers at the paranode, type (I) short linkers that link neurofilaments to the axolemma, type (II) which either seems to be composed of neurofilaments or may be their disassembled protofilaments attaching to the axolemma and type (III) slender and elongated filaments involved in cross linking neurofilaments with the axolemma. Ichimura and Ellisman (1991) proposed that this may be similar to the actin-spectrin network of red blood cell membrane cytoskeleton. In addition, on the external axolemmal aspect of the nodal and paranodal regions filaments comparable to filaments membrane-cytoskeletal linkers were observed; at the paranode they were

referred as glial membrane-cytoskeletal linkers. Ichimura and Ellisman (1991) observed that these linkers structurally connected the intermediate filaments and microtubules located deep in the axoplasm and the cytoplasm of glial cells.

I.6. Factors that control axonal calibre

There are different factors that have important functions in controlling axonal calibre including (i) the content/number of neurofilaments and their spatial arrangement (Hoffman *et al.*, 1984, 1985b, 1987; Griffin and Watson, 1988), and (ii) ensheathing glial cells (Aguayo *et al.*, 1979; Aguayo and Brady, 1984). The axonal content/number of neurofilaments depends on variations in the level of neurofilament gene expression (Lasek *et al.*, 1983; Hoffman *et al.*, 1987; Wong and Obliger, 1987), the delivery of neurofilament proteins by slow axonal transport (Hoffman *et al.*, 1984, 1985a; Parhad *et al.*, 1987; Monaco *et al.*, 1989) and the packing density of neurofilaments (Berthold, 1982; Price *et al.*, 1988a; Szaro *et al.*, 1990; Reles and Friede, 1991). Following the synthesis of the three intermediate filament proteins (NF-L, 68 kDa; NF-M, 145 kDa; NF-H, 200 kDa) in the perikaryon of a mature neuron, all three neurofilament proteins move down the axon within the slow axonal transport component [SCa] (Hoffman and Lasek, 1975; McQuarrie *et al.*, 1986) and as soon as they reach the nerve terminals, they are degraded by Ca^{2+} -activated proteases (calpains) (Roots, 1983). It has been shown that only solubilized NF-L protein self-assemble *in vitro* into intermediate filaments (Liem and Hutchinson, 1982; Gardner *et al.*, 1984; Hisanaga and Hirokawa, 1990). But studies on the human transfected adrenal carcinoma cell line SW13c1.2Vim⁻, which does not have its own cytoplasmic intermediate filament network, showed that each neurofilament triplet protein by itself

was not capable of homopolymeric assembly into filamentous arrays (Ching and Liem, 1993). Experiments using DNA transfection and mice expressing neurofilament transgenes revealed that NF-L was not able to self-assemble an extended neurofilament network in either cultured cells or transgenic oligodendrocytes which do not contain an intermediate neurofilament array (Lee *et al.*, 1993). But rather, NF-L localised into neurofilamentous punctate structures (Ching and Liem, 1993; Lee *et al.*, 1993). NF-L can be assembled with NF-M and NF-H into neurofilamentous structure *in vivo* (Liem and Hutchison, 1982; Ching and Liem, 1993; Lee *et al.*, 1993). As a result it has been suggested that *in vivo* neurofilaments may be obligate heteropolymers composed of a mixture of NF-L and NF-M or NF-H proteins (Lee *et al.*, 1993). Hisanaga and Hirokawa (1990) demonstrated that unravelled neurofilaments were formed by dialysing NF-L against a buffer containing 50 mM-NaCl without Mg^{2+} . Four 8 nm wide filaments, termed protofibrils, formed unravelled filaments. Unravelled filaments reformed from NF-L with either NF-M or NF-H subunits showed that NF-M and NF-H were incorporated evenly into each protofibril (Hisanaga and Hirokawa, 1990). Monteiro and Cleveland (1989) used transient and stable DNA transfection to induce synthesis of NF-L and NF-M in non-neuronal cultured fibroblasts when they are incorporated into intermediate neurofilament-like arrays.

In developing axons, the progressive increase in neurofilament synthesis concomitant with the observed decrease in neurofilament transport velocity has been correlated with the radial growth of the axon (Willard and Simon, 1983; Hoffman *et al.*, 1985a). During regeneration of peripheral axons (Hoffman *et al.*, 1984, 1985b; Oblinger and Lasek, 1988), decrease in neurofilament protein synthesis precedes

reduction in axonal calibre. However developmental studies of axons of retinal ganglion cells in mice have shown that phosphorylation of the carboxy-terminal tail domain of NF-M and NF-H begins at very proximal region of the axon, 150 μm from the retinal excavation (Nixon *et al.*, 1994). The increase in carboxy-terminal phosphorylation of NF-M and NF-H has been associated with an increased spacing between neurofilaments, their accumulation and an increase in axonal calibre (Nixon *et al.*, 1994) (see also pages 29-30).

It is now widely accepted that there is a correlation between neurofilament number and axonal calibre, and that regulation of neurofilaments by gene expression and axonal transport largely determines axonal size in PNS nerve fibres (Lasek *et al.*, 1983; Hoffman *et al.*, 1985b, 1987; Parhad *et al.*, 1987; Wong and Oblinger, 1987; Monaco *et al.*, 1989). But if axonal calibre is determined by neurofilament number (Friede and Samorajski, 1970; Lasek *et al.*, 1983; Lasek, 1988; Reles and Friede, 1991) then, simplistically, cytoskeletal organisation may be expected to be arranged uniformly in the axon. While, there are much greater numbers of neurofilaments in myelinated internodes than the adjacent non-myelinated region of the same fibres (Berthold and Skoglund, 1968; Hsieh *et al.*, 1994), it is also now clear that neurofilament density per μm^2 of axoplasm, obtained by dividing the number of neurofilaments by the cross section area of the axoplasm, varies from one class of axon to another; for example, in myelinated cuneate fibres of the rat the density of neurofilaments was two and half times greater than in corticospinal tract fibres (Szaro *et al.*, 1990) and neurofilament density was found to be 25% greater in large myelinated parasympathetic axons than in motor axons of the oculomotor nerve in chickens (Price *et al.*, 1988b). Indeed there are now published data which show that

the density of neurofilaments differs even along the length of single axon, for example there is an increase in the neurofilament packing density at the node of Ranvier in PNS axons (Berthold, 1982; Price *et al.*, 1988a; Reles and Friede, 1991) and in optic nerve fibres from the guinea pig (Maxwell and Graham, 1997). Thus there is increasing evidence in support of the hypothesis that there is local regulation of organisation of the axonal cytoskeleton. Additional support for this hypothetical role of neurofilaments in the radial growth of the axon was provided by the discovery of a NF-deficient mutant of Japanese quail (Yamasaki *et al.*, 1991; Ohara *et al.*, 1993). The nonsense mutation in the gene coding for the NF-L subunit (Ohara *et al.*, 1993), which forms the core of the 10 nm filament structure, caused a deficiency of neurofilaments within quail axons and the observation that the calibres in central and peripheral axons fell into smaller size classes, despite the fact that these axons had relatively thick myelin sheaths in relation to the axonal size (Yamasaki *et al.*, 1991), has been correlated with this deficiency of neurofilaments.

Early work in rat sciatic nerve by Friede and Smorajski (1970) reported different linear correlations between the number of filaments and the number of microtubules with increasing axonal calibre. Thus, as axonal calibre increases the number of neurofilaments rises faster than does the number of microtubules. Indeed the former predominate in larger fibres. Thus, based on correlation between cytoskeletal components and axonal diameter during development and regeneration, several studies (Friede and Samorajski, 1970; Friede, 1971; Lasek *et al.*, 1983; Hoffman *et al.*, 1985b, 1987, 1988a, b) it was suggested that the calibre of myelinated axons is primarily dependent upon or correlated with the number of neurofilaments.

Another factor that affects axonal calibre is the spacing between neurofilaments. It has been established that increase or decrease in NF-H phosphorylation is correlated with an increase or decrease in the interneurofilament spacing (deWaegh *et al.*, 1992; Mata *et al.*, 1992; Hsieh *et al.*, 1994; Nixon *et al.*, 1994; Tu *et al.*, 1995). Developmental studies of intact optic axons of the mouse showed that phosphorylation of the carboxyl terminus of NF-H and NF-M increase the minimum and average distance between neurofilaments (Nixon *et al.*, 1994). More recently, it has been demonstrated, in mouse axons transfected with human NF-M, that the level of phosphorylated NF-H was reduced in large-diameter CNS axons and this was associated with an increase in NF-L and the packing density of neurofilaments (Tu *et al.*, 1995). NF-H may contain the major multiple phosphorylation sites of the neurofilament triplet proteins (Lee *et al.*, 1988). Since the carboxy-terminal tail domain of NF-H protein, which forms the side-arm projections, is rich in charged amino acids, and there are multiple phosphorylation sites that may be phosphorylated to form very high amount of phosphoserine residues (Julien and Mushynski, 1982; Carden *et al.*, 1985; Zemmerman and Schlaepfer, 1986) on these side-arm projections, it has been hypothesised that local alteration in phosphorylation of NF-H could regulate the repulsive forces that influence interneurofilament spacing (Carden *et al.*, 1987; Julien *et al.*, 1988; Lee *et al.*, 1988; de Waegh *et al.*, 1992). Therefore, it seems that one function of phosphorylation is to increase the charge-based repulsion of neighbouring filaments, and as the result of that the spacing between neurofilaments is increased and consequently the axonal calibre is increased. It has been shown that in PNS axons the level of phosphorylation varies along the length of normal myelinated nerve fibres (Mata *et al.*, 1992; Hsieh *et al.*, 1994) such that at the node of Ranvier,

where axonal calibre is reduced, there is a much lower proportion of phosphorylated epitopes than in the internodal regions of the same axons (Mata *et al.*, 1992; Hsieh *et al.*, 1994). Increase in the number and spacing between neurofilaments within the internode has been correlate, hypothetically, with a greater axonal calibre, an arrangement that allows for faster conduction velocity (Waxman, 1980; Hsieh *et al.*, 1994).

However, glial ensheathment cells may also play an important role in local modulation of axonal calibre. This hypothesis developed following studies of nerve grafts in the mutant mouse Trembler (a PNS myelin-deficient mutant). In the Trembler, the average axonal calibre is smaller than in normal axons, but after transplanting normal Schwann cells into Trembler mouse nerve, the axonal calibre of the regenerated axons assumed normal dimensions (Aguayo *et al.*, 1979; Aguayo and Bray 1984). In the reverse experiment when Schwann cells from the Trembler mouse were transplanted into nerves of control mouse, the axons became thinner within the grafted segments. These data provided evidence that Schwann cells have a role in determination of axonal calibre. De Waegh and colleagues (1992) demonstrated that the level of neurofilament phosphorylation in Trembler mice sciatic nerve axons was lower than normal, but neurofilament densities, measured by counting the number of neurofilaments within a hexagonal area of $0.035 \mu\text{m}^2$ resulted an average density of 5.73/hexagon, or $164/\mu\text{m}^2$ in controls and 13.96/hexagon, or $398/\mu\text{m}^2$ in the Trembler mice. The density of neurofilaments was therefore twofold greater than normal. They hypothesised that there could be an interaction between Schwann cells and modulation of kinase/phosphatase turnover controlling neurofilament phosphorylation in the axon, to such an extent that a decrease in phosphorylation level and increase in neurofilament

density was correlated with a decrease in the average axonal calibre in the myelin deficient Trembler mouse. There is additional supporting evidence for the above hypothesis. In the rat sciatic nerve, neurofilament phosphorylation is reduced at the node of Ranvier (Mata *et al.*, 1992) where the axon is not myelinated, neurofilament numbers is reduced and their density is increased (Price *et al.*, 1988a; Reles and Friede, 1991). Nixon and colleagues (1994) in a study of the normal optic nerve in the rat suggested that myelination may have an important influence on carboxyl-terminal phosphorylation and expansion of the axon by increasing obligatory lateral spacing between neurofilaments

II. Diffuse Axonal Injury

During the last three decades both the neuropathological and neurosurgical communities have appreciated that widespread rather than focal axonal damage occurs in various form of traumatic brain injury (TBI), especially those involving acceleration and deceleration of the head. Diffuse axonal injury (DAI), which only occurs in humans, includes the widespread, scattered injury of axons throughout the brain and brain stem. Traumatic axonal injury (TAI) has long been associated with the incident of diffuse axonal injury (DAI) (Povlishock, 1992; Maxwell *et al.*, 1997b), which has been linked with post-traumatic morbidity in both human and animal models. Diffuse axonal injury occurs in human traumatic brain injuries, especially those associated with motor vehicle and auto-pedestrian accidents, assaults and falls. The severity degree of DAI varies across a spectrum of mild, moderate and severe brain injury (Peerless and Rewcastle, 1967; Oppenheimer, 1968; Adams *et al.*, 1989; Maxwell *et al.*, 1997b). In the period after injury, the injured axons undergo progressive reactive changes which

results in them dividing into two segments. The axons undergo focal axonal swelling, formation of retraction balls or axonal bulbs and eventual disconnection (Maxwell *et al.*, 1997b). The distal portion which is disconnected from the rest of axon undergoes the process of Wallerian degeneration. Weeks or months post-injury, microglial stars mark sites of earlier axonal detachment. The typical characteristic of diffuse axonal injury is that the damaged axons are scattered throughout the brain coursing among other normal, intact, unaffected axons. The degree of impairment of the brain function, with the related morbidity, depend on the number and location of the traumatically damaged axons. Various terms have been used to describe diffuse, traumatically induced axonal damage:- “diffuse degeneration of the cerebral white matter” (Strich, 1956), “shearing of the nerve fibres” (Strich, 1961), “shearing injuries of the brain” (Peerless and Rewcastle, 1967), and “diffuse brain damage of immediate impact type” (Adams *et al.*, 1977). The widely accepted present term “diffuse axonal injury” was proposed by Gennarelli and colleagues (1982) to refer to damage in the human brain. However the term has, over the last several decades, been inappropriately and inaccurately applied to numerous animal models of TBI (Maxwell *et al.*, 1997b). Thus the term, traumatic axonal injury (TAI), has been recently proposed by Maxwell *et al.*, (1997b) for description of the mode of axonal injury in animal models of human diffuse axonal injury.

II.1. A historical summary of the pathology of diffuse brain injury

Witkowski (1877) in traumatically brain injured frogs demonstrated that post-traumatic morbidity as the result of generalised damage to brain parenchyma and its intrinsic neuronal elements was independent of any vascular factors that followed the

removal of the heart and all blood vessels supplying the brain, where the frog still moved spontaneously for a short period of time. But, following a blow to the head, the frog was stunned, indicating that the response was as the result of a generalised failure of the brain parenchyma rather than its vascular supply. More recent evidence that widespread brain parenchymal damage could result from injurious events was provided by Rand and Courville (1934), who observed varicose and interrupted axons scattered throughout the brain in head injured patients, in addition to focal brain damage. Thus trauma could evoke both focal and diffuse/generalised pathological change. Over the next two decades, the clinical observations of other investigators (Russell, 1932; Greenfield, 1938; Symonds, 1943) provided additional support for the concept that neurological dysfunction could be evoked as the result of widespread injury to the neurons and their processes.

Holbourn (1943, 1945), in his novel work, showed that injury to the brain necessitated more than local contact, and suggested that widespread areas of the brain could be subjected to shearing forces generated by traumatic brain injury. Denny-Brown and Russell (1941) provided evidence that movement of the brain mass itself was a significant factor in traumatic brain injury. They observed that less force was needed to produce concussion when the head was free to move than when it was fixed. Through the use of monkeys with fitted transparent calvaria, which allowed the direct observation of the brain during trauma, the relationship between head trauma and brain deformation was investigated by Pudenz and Shelden (1946). Their work provided additional evidence for brain movement in traumatic brain injury where swirling and shifting of the brain occurred after a blow to the head. Therefore from the above experiments it became clear that brain moves within the cranium when the head

is kicked or hits the ground during traumatic injury. It was also demonstrated that such movement of the brain could result in damage to both deep and superficial parts of the brain and be responsible for posttraumatic morbidity.

II.2. Diffuse axonal injury in human traumatic brain injury

Gama was the first investigator who suggested that axon might be the locus of mechanical injury in 1835: “Fibres as delicate as those of which the organ of the mind is composed are liable to break as result of violence to the head” (Gama 1835). After one hundred years, the type of histological changes that was theorised by Gama and was predicted by Cajal’s (1928) studies were directly demonstrated by Rand and Courville (1934). The perception that the brain is subjected to widely distributed shear strains by various form of traumatic brain injury, and that these insults can be correlated with morbidity, were the background for the initiation of the clinical work of Strich (1956, 1961). Strich (1956) was the first investigator who clearly defined the occurrence of “diffuse degeneration of cerebral white matter” in her initial report from a series of patients with severe post-traumatic dementia. She examined a group of five patients who had sustained severe head injuries with significant morbidity. In the post-mortem examination of these patients there was little evidence of focal change or mass lesion formation. These patients survived from five to fifteen months after injury. Throughout their course in hospital, they were quadriparetic and unresponsive, in spite of the fact that there was no skull fracture, rise in intracranial pressure or appreciable intracranial haemorrhage. In the post-mortem examination of these patients there were no macroscopic abnormalities except enlargement of the lateral and third ventricles. Microscopic examination revealed widespread white matter degeneration along with

phagocytic invasion in the cerebrum and fibre tracts of the brain stem. Strich (1956) concluded from her findings that the morbidity seen in severe traumatic brain injury was related directly to axonal damage caused by trauma-induced shear strains similar to those described by Holbourn (1943, 1945). She subsequently examined a further fifteen patients who sustained severe traumatic brain injury and survived from two days to two years after injury. Wide spread white matter degeneration was observed in those patients who survived for a long period of time. But in those who survived for a short period of time bulbous enlargements of the axons were observed in the same region that white matter degeneration had occurred in the patients who survived for a long period of time (Strich, 1961). Based on Cajal's (1928) finding that bulbous enlargements were indicative of transected axons, Strich posited that bulbous enlargements (retraction balls) observed in traumatic brain injury provided evidence that axons were severed at the moment of impact. The clinical and post-mortem observation of Peerless and Rewcastle (1967) on 37 patients who had traumatically induced brain injuries was similar to Strich's report (1956, 1961). They also found (as Strich had found) axonal beading and retraction balls in the patients within a shorter survival time (one week) and widespread axonal degeneration concomitant with Wallerian degeneration in patients with prolonged survival time (months). They came to the same conclusion as Strich (1956) that axonal damage is the basic lesion of traumatic brain injury.

Nevin (1967) described neuropathological change in a study of 40 patients with appropriate controls using histological methods comparable to Strich's. The majority of brain-injured patients showed evidence of white matter injury, which correlated with their posttraumatic survival time. In the early phase (one week after injury) retraction

balls and phagocytic cells were present and white matter degeneration (Wallerian degeneration) was observed consistently.

Oppenheimer (1968) and Clark (1974) in a study of patients with posttraumatic survival time ranging from 12 hours to 277 days consistently identified reactive change in multiple brain sites confirming Holbourn's and Strich's suggestion that widespread parenchymal disruption is the result of the mechanical forces experienced during trauma. Oppenheimer also found that axonal damage could be detected in patients with minor head-injury who died from other causes. Thus Oppenheimer's findings and those of Peerless and Rewcastle's (1967) suggested that there was a spectrum of traumatic axonal damage ranging from mild to severe injury.

By this time it became clear to neuropathologists that diffuse axonal injury was a real entity that could be identified in traumatically brain injured patients with some direct relation to morbidity and mortality (Strich, 1956, 1961, 1970; Nevin, 1967; Peerless and Rewcastle, 1967; Oppenheimer, 1968; Clark, 1974). Following upon the initial observation of Strich (1956, 1961), Peerless and Rewcastle (1967), and Oppenheimer (1968), Adams *et al.*, (1982) characterised more fully the distribution and frequency of diffuse axonal injury in traumatically brain-injured humans. In attempting to relate the occurrence of diffuse axonal injury to specific focal lesions. They tried to find the specific loci that were involved, either macroscopically or microscopically, and relate this to their clinical observations. They also monitored the population of the patients within which DAI occurred as a primary response in the traumatic events. Adams *et al.*, (1982) hypothesised that diffuse axonal injury occurred at the moment of impact and was not secondary to any other brain damage. This was contrary to the hypothesis of earlier investigators (Evans and Scheinker,

1944; Jellinger and Seitelberger, 1970) who had suggested that such damage was secondary to associated traumatic sequelae such as hypoxia, hypotension, edema or elevated intracranial pressure.

Adams *et al.*, (1982) asserted that in its most severe form diffuse axonal injury was associated with a triad of pathological changes involving macroscopic damage in the corpus callosum, dorsolateral quadrant of rostral brain stem and microscopic change in subcortical white matter. However, Oppenheimer (1968) and Pilz (1983) showed microscopic diffuse axonal damage in patients who sustained moderate injuries without any sign of macroscopic involvement either in corpus callosum or rostral brain stem. In 1989 Adams *et al.*, described a progressive grading system of DAI, at one of which there was only microscopic evidence of axonal injury and at the other, the more severe form, there was both macroscopic and microscopic changes. This system comprised three grades of diffuse axonal injury after head injury. In grade 1 there was microscopical evidence of axonal damage in the white matter of the cerebral hemispheres, in the corpus callosum, in the brain stem, and occasionally in the cerebellum. In grade 2 there was an additional focal lesion in the corpus callosum; and in grade 3 there was also a focal lesion in the dorsolateral quadrant or quadrants of the rostral brain stem. The damage in the grade 2 and 3 can be said severe if the focal lesions appeared macroscopically.

Now nuclear magnetic resonance (NMR) imaging is used to facilitate the detection of patients who have sustained DAI. By the use of NMR, the signal changes have been recognised in deep subcortical white matter, in foci long associated with the occurrence of microscopically identifiable axonal injury (Gentry *et al.*, 1988). These

lesions are MR-detectable and believed to reflect nonhemorrhagic abnormalities, and are clearly different from those that are associated with hemorrhagic contusion.

II.3. The experimental animal models of human diffuse axonal injury

In the last two decades a substantial effort has been made to develop experimental animal models of diffuse axonal injury and to investigate the factors that are related to its pathogenesis and associated morbidity. Gennarelli and colleagues (1982) made a substantial effort to make a device to produce diffuse axonal injury in subhuman primates. They attached a helmet to an acceleration device that could produce controlled, rapid, nonimpact acceleration of the head. By using this model they generated a distribution pattern of axonal injury that was exactly spatially comparable to that observed in traumatically brain-injured patients without the generation of contusional change or mass lesion formation. Perhaps the most important conclusion of their work was the demonstration of a direct correlation between the subsequent degree of morbidity and the overall number and distribution of injured/reactive axons. A greater number of damaged axons occurred in those animals that were subjected to the highest levels of brain injury and entered a posttraumatic vegetative state than in those animals subjected to less severe damage, who demonstrated less, posttraumatic morbidity. It was important that Gennarelli *et al.*, (1982) were able to show that traumatically induced axonal injury can be produced in an animal model with an exactly comparable distribution to that found in humans, and it appeared that there was a correlation between the extent of axonal injury and the ensuing morbidity. No other animal model of human diffuse axonal injury has achieved this.

Gennarelli *et al.*, (1982) defined three grades of axonal injury in DAI. Grade 1 consisted of axonal abnormalities mainly restricted to the parasagittal white matter of the cerebral hemispheres. The axonal damage was in the form of scattered axonal retraction balls and in animals with a little longer survival, small clusters of microglia in the white matter. In Grade 2, in addition to axonal damage in the white matter of the cerebral hemispheres there was a focal lesion in the corpus callosum. In Grade 3, in addition to the damage listed above, axonal abnormalities occurred in greater numbers also in the white matter of the cerebellum, upper brainstem and the centrum semiovale.

II.4. Pathogenesis of axonal injury in animal models of human diffuse axonal injury

Although some investigators had suggested that there was a possibility of axonal stretching rather than tearing (Nevin 1967; Peerless and Rewcastle, 1967; Strich, 1970), Strich (1956, 1961) thought that shear and tensile forces of traumatic brain injury immediately tore axons throughout the brain at the moment of impact, causing the axon to retract and expel a ball of axoplasm, forming a reactive swelling or retraction ball, the traditional histologic correlate of diffuse axonal injury. However, in all models of human TAI only Maxwell *et al.*, (1993) have provided any direct evidence for tearing of axons, or disruptive axonal injury, at or very close to the time point of injury. These data were obtained after lateral head acceleration in the subhuman primate and only within the first 20 to 35 minutes after injury (Maxwell *et al.*, 1993). No experimental evidence to support the concept of direct traumatically induced axonal tearing and disconnection has been provided in any other animal model (Povlishock *et al.*, 1983; Povlishock and Becker, 1985; Povlishock and Kontos, 1985;

Cheng and Povlishock, 1988; Erb and Povlishock, 1988; Gennarelli *et al.*, 1989; Maxwell *et al.*, 1988, 1990, 1997b). Maxwell *et al.*, (1993) defined this latter form of axonal injury as non-disruptive axonal injury and it is now widely accepted to be the more frequent form of axonal injury in traumatic axonal injury (TAI) (Maxwell *et al.*, 1997b).

The hypothesis that axons tear by shear or tensile forces at the moment of traumatic brain injury, cannot be directly tested in brain-injured humans. Therefore Povlishock *et al.*, (1983) initiated a study of the pathogenesis of traumatically induced axonal injury in animals. In their first study only mild traumatic brain injury was produced in order to minimise any alteration in cerebral blood flow, blood brain-barrier status and vascular reactivity that might also contribute to the genesis of the observed axonal abnormalities. To generate mild brain-injury fluid percussion of the intact dura mater was used in cats. In this experiment anterograde tracer such as horseradish peroxidase conjugated to wheat germ agglutinin was used to test the hypothesis that if the axon tears at the moment of injury, then those axons laden with anterogradely transported protein would rupture and expel a mass of protein (peroxidase)-containing axoplasm that could be visualised by histochemical methods at both the light and electron microscopic level. Povlishock found no evidence for direct axonal tearing and extrusion of axoplasm. But, using light microscopy, it was observed that a focal impairment of the anterograde axoplasmic transport of the protein tracer along the axon's length was evoked by TAI (Povlishock *et al.*, 1983). Both light and electron microscopy provided evidence for intra-axonal pooling of the anterograde tracer, horseradish proxidase, within one hour of injury. This pooling occurred at a singular locus rather than multiple loci along the length of a particular axon. At this time the

site of peroxidase pooling still was in continuity with the proximal and distal portions of the axon. The axolemma and the myelin sheath were not disrupted. Although this is the word used by Povlishock *et al.*, (1983), later evidence has shown that the axolemma was “perturbed” or became permeable to tracers such as HRP (see pages 49-50). There was no evidence of tearing of the axons at this time point. The sites of tracer pooling typically occurred in paranodal and internodal regions, and there was little involvement of the node of Ranvier. These sites showed tubular and vesicular profiles of smooth endoplasmic reticulum that contained peroxidase together with the other organelles, and accumulation of neurofilaments. With greater survival time, 2 to 3 hours after injury, continued anterograde delivery of horseradish peroxidase-containing tubular and vesicular profiles and other organelles to the site of axonal injury, lead to an increase in the calibre of the axon. The expanded segment of the axon eventually underwent lobulation such that axonal diameter ranged from 10 to 12 μm . 4-6 hours after trauma, a constriction formed between the lobes of lobulated axons such that a narrowed thread of protein now connected two segments together. At this point the reactive swelling-containing proximal axonal segment detached from its distal counterpart and typically this occurred within the first 6 to 12 hours following injury. During this phase of continued expansion, anatomical disconnection was hypothesised to have occurred between the swollen proximal segment in continuity with the sustaining soma and the more distal axonal shaft. However, the actual process of disconnection had not been clearly described. Maxwell and Graham (1996), however, provided some morphological evidence for the process of axonal disconnection between 2 and 4 hours after stretch-injury to guinea pig optic nerve. They observed that in the absence of accumulation of membranous organelles and

axonal swelling in some axons the neurofilament linearity was replaced by a non-linear/granular substructure. This was associated with interdigitation of axolemmal processes which were separated by an irregular intra-axonal space and disruption of the myelin sheath (Maxwell and Graham, 1996). Compaction of neurofilaments associated with axolemma and myelin sheath disruption, and separation of the myelin sheath from the axolemma forming a “balloon-like” expansion was observed in some larger fibres in the optic nerve of the guinea pig 4 hours after stretch-injury (Jafari *et al.*, 1997). Another alternative for axonal disconnection is that at the time of axotomy the axolemma disrupts and recoalesces to form two discrete segments of the axon which are frequently encompassed by the same continuous myelin sheath (Erb and Povlishock, 1988; Povlishock, 1992, 1993). In addition to light microscopic observations, frankly separated axon segments with organelle accumulation, covered by recoalesced and distended axolemma in an overlaying continuous thin myelin sheath, had been observed under the electron microscope after severe TBI to cats (Erb and Povlishock, 1988). Shortly after axonal separation, the myelin sheath also disrupts and recoalesces to encompass the proximal expanded segment. Between 12 to 24 hours of injury the disconnected, terminal bulbs consisted of a central core containing numerous neurofilaments and some microtubules surrounded by numerous mitochondria and peroxidase-laden tubular and vesicular smooth endoplasmic reticulum. These ball-like swellings, achieve a diameter of 15-40 μm (Povlishock *et al.*, 1983). 24 hours after TAI in the cat the same type of peroxidase-laden swellings were observed (Povlishock *et al.*, 1983). The distal segment underwent Wallerian degeneration and seemed to withdraw from the locus of detachment. After detachment the tip of the proximal segment continued to expand as tubular and vesicular profiles

of smooth endoplasmic reticulum and other organelles continued to be delivered, via anterograde transport, to the site (Povlishock *et al.*, 1983). The peroxidase containing tubular and vesicular profiles of smooth endoplasmic reticulum, together with other related organelles, increased in number surrounding an expanded and disorganised neurofilamentous core which increased the expansion of the tip of proximal segment of the axon (Povlishock *et al.*, 1983). With further survival time this expansion was associated with the compensatory distension and thinning of the overlying myelin sheath, therefore giving rise to the so called “mature” reactive axonal swelling, retraction ball or, using the recently established terminology (Maxwell *et al.*, 1997b), axonal bulb. Thus no morphological evidence has been provided to support the hypothesis that direct traumatically induced axonal tearing or renting occurs in the majority of injured axons after TBI. But rapid local accumulation of vesicular and tubular profiles of smooth endoplasmic reticulum as the result of localised impairment of fast anterograde transport system, are known to participate in the process of disconnection (Grafstein and Forman, 1980).

The above described axonal swelling and its correlation with focal impairment of axonal transport is supported by comparable observations in studies of peripheral nerve transection and ligation (Martinez and Friede, 1970; Griffin *et al.*, 1977).

The occurrence of focal axonal swelling and lobulation leading to axonal disconnection as the result of focal impairment of axoplasmic transport also has been observed in the sequence of reactive axonal change in animal models of more severe traumatic brain injury (Erb and Povlishock, 1988; Maxwell *et al.*, 1988). In severe traumatic brain injury axonal disconnection, currently termed secondary axotomy, occurred in a much shorter period of time (1-2 hours) and focally limited

multilobulated axonal swellings were observed instead of single lobular axonal swelling (Erb and Povlishock, 1988).

As has been described above the progressive events leading to axonal disconnection and full development of retraction balls in the proximal segments takes 6-24 hours to occur (Povlishock *et al.*, 1983). Apart from some rare cases in which disconnection takes place in less than 2 hours, in the majority of cases frank disconnection requires at least 6 hours, and the most severe injuries results in the most rapid progression to axonal disconnection (Erb and Povlishock, 1988). But more recent work has resulted in a re-appraisal of this general time course. It is now clear that species differences and injury models that are used also have influence on the speed of disconnection (Povlishock, 1993; Maxwell *et al.*, 1997b). Thus it has fairly recently been suggested that the progression of axonal change is faster in rodents, sometimes occurring within one hour of injury, than in cats (Povlishock, 1993). For example, that the time to axonal disconnection after using fluid-percussion or cortical impact models of traumatic brain injury in rats (Yaghmai and Povlishock, 1992) is about two hours. In the cat, diffusely damaged axons were mostly found in the brain stem and diencephalon after fluid-percussion injuries of differing severity (Povlishock *et al.*, 1983; Erb and Povlishock, 1988; Yaghmai and Povlishock, 1992). Therefore, in comparison with rats the time to disconnection in cats was found to be longer, with axonal disconnection occurring within 6-12 hours in minor head injury (Povlishock *et al.*, 1983) and between 2 and 6 hours after severe TBI (Erb and Povlishock, 1988). Further, after acceleration and deceleration to the head of miniature swine in the coronal plane retraction balls (axonal bulbs) were observed at 6 hours (Ross *et al.*, 1994).

There is now considerable evidence that secondary axotomy occurs more rapidly in rats, cats and miniature swine than in humans (Polvishock, 1992, 1993; Grady *et al.*, 1993; Christman *et al.*, 1994). Early neuropathological observations suggested that retraction balls are not seen unless traumatically brain-injured humans survive for at least several hours post-injury (Pilz, 1983; Vanezis *et al.*, 1987; Adams *et al.*, 1989; Grady *et al.*, 1993; Christman *et al.*, 1994). However, the recent development of more sensitive labelling techniques utilizing immunocytochemical labelling for β -amyloid precursor protein (β -APP) (Gentleman *et al.*, 1993, 1995; Sherriff *et al.*, 1994; Blumbergs *et al.*, 1994, 1995; McKenzie *et al.*, 1996) has allowed the demonstration of injured/damaged, but not disconnected, axons within 1.75-2 hours of injury (Blumbergs *et al.*, 1994; McKenzie *et al.*, 1996) and observation of axonal bulbs 3 hours after non-missile head injury (Mackenzie *et al.*, 1996). Thus it may be concluded that the process of axonal perturbation that results in axonal swelling and disconnection in experimental animals does also occur in humans. But there are differences in the detail of the time course of these axonal responses between man and experimental animals. Further, it is a characteristic of the diffuse type of axonal damage that damaged axons occur in close spatial relation to normal or intact axons in the same tracts or bundle of axons (Povlishock *et al.*, 1983; Gennarelli *et al.*, 1989), and it is impossible to tear some axons mechanically within a tract while keeping the adjacent axons and vasculature intact, direct axonal tearing was not the cause of genesis of the events in the process of reactive axonal change.

Early results of changes in the metabolism of glucose in the somata of neurons whose axons were undergoing reactive axonal change demonstrated no significant change in the metabolism of glucose detected by ^{14}C -2-deoxyglucose (Hayes *et al.*,

1984, 1988) autoradiography. On the other hand Gennarelli *et al.*, (1989), using the same technique on horizontal sections through the ganglion cell layer, showed that there was a 13% increase in glucose metabolism in the cell bodies after stretch-injury to the guinea pig optic nerve. It has been also observed that reactive axonal change commonly occurred with no evidence of concomitant focally related neuronal somatic or dendritic changes in vestibular, reticular and red nuclei of the brain after fluid-percussion head injury in cats (Povlishock, 1986). In a further study using the same injury model, the cat's visual system also showed that there was no concomitant alteration in the surrounding parenchyma and vasculature of the lateral geniculate body in relation to reactive axonal swelling (Cheng and Povlishock, 1988). However, Povlishock *et al.*, (1986) observed that some isolated neurons showed a chromatolytic response in relation to reactive axonal change. But this chromatolysis occurred in neurons whose axons were injured proximal/close to the cell soma (Povlishock, 1986). Therefore, this somal chromatolysis appeared to be a secondary response to initial axonal injury rather than a primary incident (Povlishock, 1986; Maxwell *et al.*, 1994c). Maxwell *et al.*, (1994c) have demonstrated that about 30% of medium and large size retinal ganglion cells underwent central chromatolysis between 3 and 7 days of stretch-injury to guinea pig optic nerve. 3 days post-injury Nissl substance was located at the periphery of cell and the nucleus was eccentric. By 7 days, the size of chromatolytic cells was reduced together with almost complete loss of rough endoplasmic reticulum and ribosomes. Between 7 and 14 days these cells degenerated, and some cells were lost by 14 days after stretch-injury. There was not, however, any morphological change in the small ganglion cells or small neurons. Maxwell *et al.*, (1994c) also provided morphological evidence for regeneration or recovery in a small proportion of

large retinal ganglion cells which showed an increase in somal and nucleolar size, together with reformation of rough endoplasmic reticulum.

The advantage of use of the visual pathway in fluid-percussion injury is that the ganglionic neurons whose axons form the optic nerves are protected from a direct mechanical effect in percussion injury. Therefore any possibility of the soma being directly affected by the mechanical insults that cause reactive axonal change may be excluded, and axonal injury cannot be attributed to changes in the neuronal somata. For this reason reactive axonal changes observed in the optic tract are believed to be directly related to primary focal axonal injury, not the cumulative effect of neuronal somatic perturbation (Cheng and Povlishock, 1988). Comparable findings have also been reported in later studies of several different animal models of traumatic insult (Gennarelli *et al.*, 1984, 1989; Maxwell *et al.*, 1988, 1991a, 1994), notably in the subhuman primate model of acceleration injury (Gennarelli *et al.*, 1984) where comparable impairment in axoplasmic transport was observed. Also, using a model of stretch-injury of the optic nerve, a repertoire of intra-axonal changes similar to those after fluid-percussion has been reported (Gennarelli *et al.*, 1989; Maxwell *et al.*, 1990, 1991a, 1994a, b, 1995; Jafari *et al.*, 1997). Further, this latter model of axonal injury allows more detailed analysis of structural alternations in axons after injury than do studies where brain damage is diffuse, and the conditions resulting in axonal injury can not be characterised with precision.

Early work on models of TBI/TAI suggested that focal tissue ischaemia and/or disruption of the blood-brain barrier evoked by the traumatic incident caused intra-axonal changes (Evans and Scheinker, 1944; Jellinger and Seitelberger, 1970; Jellinger, 1977). But more recent observations of brain parenchyma and vasculature in

the regions undergoing the process of traumatically induced axonal change have provided no evidence for microvascular alteration after traumatic injury (Povlishock and Becker, 1985; Povlishock and Kontos, 1985; Povlishock, 1986). Rather, axons in the process of reactive axonal change were found without any evidence of focally related alteration of adjacent somata, dendrites, axons and microvessels in the same field (Povlishock, 1985; Povlishock, 1986; Povlishock, 1991). Although Maxwell *et al.*, (1988) declared that all reactive axons took place within 4.5 mm of a haemorrhagic focus in the corpus callosum after angular head acceleration; other experimental studies, in a variety of types of experimental animals, have not demonstrated that reactive axonal change is associated with changes in blood flow (DeWitt *et al.*, 1986; Jenkins *et al.*, 1986; Yuan *et al.*, 1988; Yamakami and McIntosh, 1989). Use of ^{14}C -labelled iodoantipyrine autoradiography in a series of studies after TBI provided no evidence for ischaemia at foci that showed reactive axonal change (Povlishock, 1990, 1991). Maxwell *et al.*, (1992) found that a small number of blood vessels (approximately 5%) in all parts of the brain showed posttraumatic extravasation of blood. However, the vast majority of blood vessels were not associated with petechial haemorrhage one hour after lateral head acceleration in subhuman primates. Examination of widespread parts of brain after lateral head acceleration demonstrated that structural changes in blood vessels were not limited to the site of occurrence of traumatic axonal injury or petechial haemorrhage (Maxwell *et al.*, 1992). Neither has evidence for extravasation or blood vessel tearing been found after stretch-injury to guinea pig optic nerve (Maxwell *et al.*, 1991b), nor was evidence for blood flow abnormalities at foci of reactive axonal change obtained in moderate traumatic brain injury (Povlishock, 1991; Povlishock *et*

al., 1992). Despite the fact that cerebral blood flow was reduced in some regions of the brain (Povlishock, 1991; Povlishock *et al.*, 1992) the reduction in blood flow never reached ischaemic levels. Further, all blood flow returned to normal or near normal levels 6 hours after injury. Use of intravascular peroxidase or immunological labelling against blood serum provided no evidence for a correlation between the sites where blood-brain barrier permeability was altered and reactive axonal change occurred (Povlishock, 1991; Povlishock *et al.*, 1992). Reactive axons could be found both at foci where there was an opening of the blood-brain barrier or at foci with an intact barrier.

Further studies in severe traumatic brain injury in cats did not provide evidence for a correlation between damaged axons and petechial hemorrhage (Erb and Povlishock, 1988). Therefore the occurrence of reactive axons at foci either with or without damaged microvessels strongly suggests that reactive axonal change does not take place as a result of direct tissue tearing and microvasculature abnormalities.

II.5. Subcellular events that initiate steps in genesis of reactive axonal change

In spite of the fact that many investigators have made a great effort in providing valuable information on the development of axonal change after trauma, the subcellular events that lead to axonal abnormalities have still not been described. Two of several reasons for this shortcoming are:- first, the diffuse or widespread nature of the axonal injury provides a difficult situation for proper sampling procedures which are more difficult when highly focal and discrete intra-axonal changes are examined. Second, although the use of anterograde tracer HRP (horseradish peroxidase) is helpful in the detection of alteration in axonal transport since it migrates anterogradely

by fast axonal transport (Mesulam and Mufson, 1980), the intra-axonal tracers are generally limited to tubular and vesicular pools, and, in early work, they did not reveal the precise site of any intra-axonal abnormality until relatively significant pooling of organelle population had occurred (Povlishock *et al.*, 1983). However, more recent studies have demonstrated that HRP (which is normally excluded by the axolemma) appeared in injured axons and indicated a focal change in their axolemmal permeability 5 minutes (Pettus *et al.*, 1994; Povlishock *et al.*, 1995; Pettus and Povlishock 1996) and even immediately (Povlishock *et al.*, 1997) after traumatic brain injury both the cat and rat.

To initiate the investigation of the initial intra-axonal changes, many investigators focused their studies on the calcium hypothesis of injury, derived from work generated in the fields of spinal cord and peripheral nerve injury (Balentine and Spector, 1977; Schlaepfer and Micko, 1978; Mata *et al.*, 1986; Schlaepfer, 1974, 1987; Balentine, 1978, 1980, 1985, 1988). As hypothesised in the Central Nervous System Trauma Status Report (Balentine, 1985; Povlishock, 1985), shear and tensile forces occurring in both brain and spinal cord trauma are associated with stretching of the axolemma. Hypothetically, this stretching increases axolemmal permeability and allows the influx of toxic levels of intra-axonal calcium, which under normal conditions is maintained at a 10,000 times greater concentration in the extra-axonal environment. This transaxolemmal concentration gradient is maintained by the operation of membrane-bound Na^+ - Ca^{2+} antiporters (that is the transport of two components across membrane in opposite direction) and Ca^{2+} -activated ATPases, together with sequestration by mitochondria, endoplasmic reticulum and Ca^{2+} -binding proteins so that the value of intra-axonal calcium normally is maintained at about 10^{-7} M (Siesjö and Wieloch,

1985). Each of the above mentioned processes requires to some extent an energy source (Siesjö and Wieloch, 1985). It has been hypothesised that under resting conditions most of the net Ca^{2+} extrusion from the axon is carried out by the ATP dependent axolemmal pump (Waxman and Ritchie, 1993; Maxwell *et al.*, 1995). The activity of $(\text{Ca}^{2+}\text{-Mg}^{2+})\text{-ATPase}$, together with cell adhesion molecules such as the neuronal cell adhesion molecule ecto- $\text{Ca}^{2+}\text{-ATPase}$, has been demonstrated using cytochemical techniques (Maxwell *et al.*, 1995). They demonstrated influx of calcium and alteration in labelling/or loss of membrane pump and ecto- Ca^{2+} -activity after stretch-injury/non-disruptive axonal injury to guinea pig optic nerve. They also suggested that these events may take part in the ensuing pathological cascade after TAI to optic nerve fibres.

It has been hypothesised that the level of intracellular calcium is elevated by influx of calcium which in turn activates neutral protease (calpains), that cause neurofilament dissolution, and as a result of this, disorganisation and local collapse of axoplasm (Schlaepfer and Bunge, 1973; Schlaepfer and Micko, 1978; Schlaepfer and Hasler, 1979; Schlaepfer and Zimmerman, 1985; Schlaepfer *et al.*, 1985; Mata *et al.*, 1986; Schlaepfer, 1987). Studies of the proteolytic activity of calcium-activated neutral proteases, now referred to as calpains, on proteins such as extracted neurofilament triplet proteins, tubulin and microtubule associated proteins in isolated microtubules have shown that the activity of these calpains is stimulated by increased calcium concentration (Ishizaki *et al.*, 1983; Kamakura *et al.*, 1985; Billger *et al.*, 1988; Cottin *et al.*, 1991; Banay-Scwartz *et al.*, 1994). Two types of calpain (mM calpain and μM calpain) are found in axons (Job *et al.*, 1981; Kamakura *et al.*, 1985; Billger *et al.*, 1988; Cottin *et al.*, 1991). These calpains degrade neuronal cytoskeletal proteins such

as microtubule associated protein 2 (MAP2), actin, spectrin and neurofilaments (Murachi, 1989). Both μM calpain and mM calpain and the enzyme inhibitor, calpastatin, coexist widely in mammalian tissue including brain tissue (Murachi *et al.*, 1981; Murachi, 1983). It has been demonstrated that calpastatin exists in neurons, glial cells, axons and weakly in the myelin sheath (Kamakura *et al.*, 1992). The interaction of intracellular calcium and the endogenous calpain inhibitor, calpastatine, regulate the activities of calpains (Murachi, 1989). μM calpain is located primarily in the neuron while mM calpain is more abundant in glial cells (Hamakubo *et al.*, 1986; Murachi, 1989). The two types of calpain have also been recognised in mouse optic nerve (Nixon *et al.*, 1986). μM calpain has a high affinity for calcium and is activated by μM (less than 0.1 mM) concentration of calcium, whereas mM calpain requires at least 0.20 mM calcium for activation (Glass *et al.*, 1994) and has a low affinity for calcium (Schlaepfer and Zimmerman, 1985). Purified neurofilaments are degraded by μM calpain and mM calpain with apparent K_m values of 3.9×10^{-8} , 4.4×10^{-8} , 8.2×10^{-8} M for NF-70, NF-150 and NF-200 respectively (Zimmerman and Schlaepfer, 1982). It has also been demonstrated that μM calpain is more specific for targeting its substrate with the neurofilament side-arm being the primary target (Ishizaki *et al.*, 1983; Kamakura *et al.*, 1985). Kamakura and colleagues (1985) demonstrated that 0.1 mM Ca^{2+} did not activate mM calpain, but μM calpain became 100% activated in the presence of 0.1mM Ca^{2+} . The neurofilament triplet proteins were degraded in the order of 160 kDa, 68 kDa, and 200 kDa neurofilaments subunits by μM calpain from chicken muscle. 160 kDa also was degraded faster than the other subunits by both mM and μM calpains. 200 kDa neurofilament subunit was degraded more easily and greater than 68 kDa by purified mM calpain from chicken muscle and cathepsin B on

long-term incubation. But bovine liver purified natural μM calpain degraded 200 kDa more extensively than 68 kDa neurofilament subunit on long-term incubation (Kamakura *et al.*, 1985).

Altered axonal permeability has been found in crushed or sectioned peripheral nerve, crushed spinal cord, traumatic brain injury and in stretch-injury in the optic nerve. This was associated with intra-axonal influx of various extracellular confined substances, not only calcium but also various extracellularly confined macromolecules (Schlaepfer, 1974; Balentine and Spector, 1977; Kristensson, 1978; Olsson *et al.*, 1978; Malmgren and Olsson, 1979; Schlaepfer *et al.*, 1985; Mata *et al.*, 1986; Balentine, 1985, 1988; Pettus *et al.*, 1994; Pettus and Povlishock, 1995, 1996; Maxwell *et al.*, 1995; Maxwell, 1996), consistent with a traumatically induced nonselective permeability change. In moderate traumatic brain injury local axolemmal permeability to extracellularly confined peroxidase was recognised. This peroxidase passage was associated with local mitochondrial abnormalities and increased packing of neurofilaments which led to delayed progression of reactive axonal change (Pettus *et al.*, 1994). The findings of Pettus and Povlishock (1995) suggested that, in some cases, a persisting change in axolemmal permeability can occur as the result of traumatic brain injury. The same authors in continuation of their 1996 studies suggested that axolemmal permeability triggers a rapid, yet persisting general cytoskeletal change most likely linked to local ionic dysregulation. The demonstration of altered pyroantimonate distribution in stretch-injured myelinated fibres and altered distribution of both membrane pump and ecto- Ca^{2+} -ATPase activity (Maxwell *et al.*, 1995) provided the first cytochemical evidence in support of the hypothesis (Adams *et*

al., 1991) that posttraumatic influx of calcium ions occurred in stretch-injured and nondisruptively injured nerve fibres.

Maxwell *et al.*, (1991a), in the optic nerve of guinea pig subjected to rapid, but controlled stretching (Gennarelli *et al.*, 1989), demonstrated that the initial axonal damage occurred at the node of Ranvier. In this study, routine ultrastructural analysis within the first hour of survival revealed that the earliest morphological change, 15 minutes after stretch-injury, was the development of a membrane-bound, nodal bleb, containing a small quantity of axoplasm, extending from a damaged node of Ranvier (Maxwell *et al.*, 1991a). It was found that, these nodal blebs occur at a single locus along the axon's length and were most frequent at 15 minutes, less frequent at 2 and 6 hours and not present at later survivals after injury (Maxwell *et al.*, 1991a). As the nodal blebbing became less frequent by the end of the first hour pooling of vesicular and tubular profiles of smooth endoplasmic reticulum occurred, in a fashion consistent with that earlier described by Povlishock *et al.*, (1983), within the related paranodal and internodal regions. Loss of the nodal subaxolemmal density was noted at sites of ballooning of the axolemma at damaged nodes (Maxwell *et al.*, 1991). In the damaged node of Ranvier there was also a limited local disorganisation of neurofilaments associated with numerous membranous profiles of smooth endoplasmic reticulum in the axoplasm. But no local dissolution of the cytoskeleton was observed (Maxwell *et al.*, 1991a). There was a marked accumulation of membranous organelles in the paranodal and internodal regions between 2 and 6 hours after the injury, and this was associated with disruption of the myelin sheath and glial-axonal junctions. The formation of typical classic, degeneration balls or axonal bulbs and axotomy occurred between 24-72 hours after stretch injury. The structural similarities found in both

experimental injuries to the brain and in man make it reasonable to assume that the progression of axonal changes observed in experiments also occurs in man (Povlishock, 1992; Maxwell *et al.*, 1997b).

Based on these findings Maxwell *et al.*, (1991a) argued that the node was a principal and initiating site for traumatically induced reactive change. They suggested that traumatic perturbation of the axolemma or related axoplasm of the nodal region somehow impaired axoplasmic transport resulting in genesis of reactive axonal swellings in related paranodal and internodal regions. The hypothesis about nodal changes and calcium influx was substantiated by Maxwell (1996) and Maxwell *et al.*, (1995).

II.6. Factors affecting on the genesis of experimental axonal injury

Several factors may be involved in the pathogenesis of traumatically induced reactive axonal change. The species studied is one of the factors that influences the temporal course of axonal swelling leading to detachment (see also pages 44-45). The course of axonal swelling and detachment in rodents is within 2-4 hours after injury (Povlishock, 1992; Yaghmai and Povlishock, 1992), while in humans axonal swelling and detachment occurs over a period up to 12 hours (see also page 45) (Povlishock, 1992, 1993; Grady *et al.*, 1993; Christman *et al.*, 1994) but may also be shown either at 88 hours (Gradey *et al.*, 1993; Christman *et al.*, 1994) or 99 days (Blumbergs *et al.*, 1994) after injury. In spite of the fact that the period of axonal swelling and disconnection in higher species is longer, in all species some axons are observed in the state of disconnection while occasional fibres in the same field demonstrate the initial phase of reactive axonal change (Povlishock, 1992; Grady *et al.*, 1993). It

may therefore be posited that there is continuing recruitment of injured or reactive axons over a long time course after human blunt head injury.

It has been suggested by Maxwell *et al.*, (1993) that traumatically induced axonal change may involve a varied pathogenesis. In their study on axons from the corpus callosum of three subhuman primates, within the first hour after severe traumatic brain injury, direct tearing of the axon was recognised within the first 20 to 35 minutes of injury. Axonal fragmentation occurred both in nodal/paranodal and internodal regions and was frequent at 20 minutes, less frequent at 35 minutes after injury and generally not present by one hour. Since cytoskeletal dissolution was followed by swelling of the axon, and was accompanied by a flocculent precipitation of axoplasm rather than the accumulation of organelles, Maxwell *et al.*, (1993) suggested that disruption of the axolemma in the node/paranode junction and in the internode allowed for the influx of calcium. This influx of calcium was hypothesised to reseal the membrane by the activation of phospholipase A₂ within one hour of injury as, for example has been demonstrated in nerve fibres of *Periplaneta americana* (Yawo and Kuno, 1983), and simultaneously the influx of Ca²⁺ could activate μ M calpain and mM calpain (Murachi, 1983) to dissolve the axonal cytoskeleton. Maxwell *et al.*, (1993) also found isolated organelle-laden swellings in the same tissue. They hypothesised that this second type of swelling occurred as the result of impaired axoplasmic transport as described by other investigators (Povlishock *et al.*, 1983; Erb and Povlishock, 1988; Gennarelli *et al.*, 1989; Maxwell *et al.*, 1991a; Povlishock, 1992, 1993). The finding of two different response by two subgroups of injured axons suggested to Maxwell *et al.*, (1993) that there was the possibility that two different events occurred in traumatically injured axons: one group designated as nerve fibres undergoing primary axotomy

associated with direct axolemmal disruption and calcium influx and the other with the designation of secondary axotomy concomitant with subtle intra-axonal change, resulting in impairment of anterograde transport and the formation of axonal swellings. Povlishock, (1992) and Maxwell *et al.*, (1997b) suggested that primary axotomy is more likely to occur or have occurred in those animals and patients who sustained the severest form of traumatic insult. The differences in the pathological processes obtained from the work in Maxwell's group and Povlishock's group may be influenced by fibre size, nature of injury and/or the species used. All changes that were described by Maxwell *et al.*, (1991a, 1993) were confined to small calibre axons. The axolemmal disruption observed by Maxwell *et al.*, (1993) in primary axotomy were only from small calibre axons ranging from 0.50 μm to 1.50 μm and there was no evidence of abnormality in the larger size axons, while observations made by Povlishock's group were limited to long tract and large calibre axons (Povlishock *et al.*, 1983; Erb and Povlishock, 1988; Pettus *et al.*, 1994; Pettus and Povlishock, 1995, 1996; Povlishock and Pettus, 1996; Povlishock *et al.*, 1995, 1997). Nodal axolemmal blebbing also was observed in small calibre axons (Maxwell *et al.*, 1991a).

It is established that there are anatomical differences between small and larger calibre axons in normal/uninjured animals. For example in uninjured, small PNS axons the internodal distance is short and the proportion of microtubules is higher than that of neurofilaments (Friede and Samorajski, 1970). More recent data has shown that the number of microtubules in the smallest myelinated axons in the guinea pig optic nerve is greater than the number of neurofilaments (Jafari *et al.*, 1997). Large calibre axons, however, have a relatively long internodal length and the proportion of neurofilaments is higher than microtubules, with neurofilaments outnumbering microtubules by as

much as 10:1 (Friede and Samorajski, 1970; Hoffman *et al.*, 1984, 1985b; Jafari *et al.*, 1997). The numbers of neurofilaments and microtubules increase with axonal calibre in myelinated and nonmyelinated fibres in the rat sciatic nerve, but the number of microtubules increase at a relatively slower rate because of the relatively lower concentration of microtubules with increasing fibre size (Friede and Samorajski, 1970; Reles and Friede, 1991). More recent from CNS fibres in the guinea pig optic nerve indicate an exactly comparable trend, acknowledging that the sizes of fibres differ markedly between the PNS and CNS (Jafari *et al.*, 1997). Detailed analysis mouse sciatic nerve fibres showed that there were often more microtubules at the node of Ranvier than at the internode, and that the density (that is the number of microtubules per μm^2 area of axoplasm) of microtubules was greater at nodes (Reles and Friede, 1991). Unlike the situation at the internode, at the node the number of microtubules increased with increasing axonal calibre, such that the increase in the number of microtubules was slightly greater than the increase in the number of neurofilaments (Reles and Friede, 1991). Further, it has been found that neurofilaments in cuneate axons of rat were two and half fold more dense (the density was obtained by counting the number of either neurofilaments or microtubules per μm^2 area of axoplasm) than neurofilaments in dorsal corticospinal axons, but microtubules in cuneate axons were much less dense than neurofilaments in the same axons and than microtubules in corticospinal tract axons (Szaro *et al.*, 1990).

It has not been clarified yet in man and animal models of diffuse axonal injury why some axons are injured while the others that run in the same course in the immediate vicinity remain intact. It has been suggested, both in man and animals, that the following axons are likely be susceptible to damage: those axons that change their

anatomical direction to reach their target nuclei, ones that decussate and those axons that turn obliquely around blood vessels in their course (Povlishock, 1992, 1993; Yaghmai and Povlishock, 1992). At such points axons are under maximum tensile strain, and neurofilaments and microtubules slide over one another resulting a viscous separation of the axoplasm (Brown and Lasek, 1990). If this happened, it has been posited, the sliding of neurofilaments and microtubules could break neurofilament side-arms resulting in disorganisation and increased packing of neurofilaments.

II.7. Pathological changes in the cytoskeleton

II.7.a. Neurofilaments

Various antibodies have been used to assess intra-axonal neurofilament subunit change following traumatic brain injury. But there is also some evidence for interspecies differences, for example more retraction balls (axonal bulbs) were more densely labelled with antibodies against non-phosphorylated epitopes of NF-H and NF-M subunits than phosphorylated ones after fluid-percussion injury or cortical impact in rats (see Ross *et al.*, 1994), while axonal bulbs were more densely labelled with antibodies against phosphorylated epitopes of NF-H and NF-M subunits in human, non-human primates and in miniature swine (Ross *et al.*, 1994). It has been demonstrated that antibodies targeted to the NF-L (70kDa) subunit label reactive axons in a wide range of species ranging from humans (Grady *et al.*, 1993; Christman *et al.*, 1994) to rodents (Yaghmai and Povlishock 1992). In studies of fluid-percussion brain injury in rats and cats, antibodies were targeted to the NF-L (68 kDa), NF-M (150 kDa) and NF-H (200 kDa) neurofilaments subunits at various times of injury prepared for light and electron microscopy. This technique revealed no evidence of

focal intra-axonal neurofilament dissolution (Yaghmai and Povlishock, 1992). It was found that there were localised increase in 68 kDa subunit immunoreactivity within the first 15 minutes of TBI (Povlishock, 1992). By this time electron microscopic observation revealed that there were infoldings of the overlaying axolemma (Povlishock, 1992). In a light microscopic observation Yaghmai and Povlishock (1992) demonstrated that one hour post-injury antibodies to 68 kDa NF subunit revealed intense immunoreactivity within axonal segments undergoing reactive axonal change, and EM microscopic analysis showed that this increased immunoreactivity was associated with an increased number of 68 kDa-immunoreactive filaments but that the majority of them still were parallel to the long axis of the axon. The immunoreactivity against phosphorylated 200 kDa epitope in the event of reactive axonal change appeared reduced from levels in the control situation. Within 2-6 hours of injury 68 kDa immunoreactive neurofilaments began to withdraw from the focus of injury displaying an alignment oblique to the long axis of the axon (Yaghmai and Povlishock, 1992). These neurofilaments showed an increased disorganisation and over time became internalised to the core of axon and were associated with accumulation of organelles at the periphery forming the expanding organelle cap as described in various studies from Povlishock's group (Povlishock *et al.*, 1983; Povlishock and Kontos, 1985; Erb and Povlishock, 1988). Six hours after injury, in spite of the fact that most of reactive axonal segments demonstrated a frank disconnection from their distal segment, there were, however, some swollen reactive axonal segments which remained in continuity with the rest of axon. From their findings Yaghmai and Povlishock (1992) suggested that the process of reactive axonal change and disconnection is not necessarily homogeneous in all reactive axons. For

example, two different cytoskeletal responses were observed after moderate/severe fluid-percussion brain injury in cats (Pettus and Povlishock, 1996). Immunoreactive axonal segments, at the ultrastructural level, showed tightly packed neurofilaments which were aligned randomly in relation to the long axis of the axon cylinder. The 200 kDa subunit immunoreactivity was limited to the periphery of the reactive axon, whereas 68 kDa subunit immunoreactivity was localised centrally and 150 kDa subunit immunoreactivity showed a diffuse and generalised distribution (Yaghmai and Povlishock, 1992).

Hall and Lee (1995) demonstrated that concomitant with the loss or reduction of neurofilament side-arm staining, using specific monoclonal antibodies, neurofilament rod domains were stained with rod-specific monoclonal antibodies nearest to the lesion site both in proximal and distal stumps 12 hours after axotomy, by spinal cord transection, of lamprey spinal axons. According to the study of axotomy of Mauthner axons in the lamprey, there is a large influx of Ca^{++} during the first day postaxotomy (Borgens *et al.*, 1980; Strautman *et al.*, 1990). Hall and Lee (1995) suggested that activation of proteases by Ca^{2+} influx could be at least partly responsible for neurofilament side-arm proteolysis/collapse since Goldstein and colleagues (1987) had provided some evidence in mammalian systems for a mechanism whereby neurofilament dephosphorylation leads rapidly to proteolysis. Therefore, it might be hypothesised that side-arm proteolysis occurs as the result of dephosphorylation or activation of protease by Ca^{2+} influx, or both. Hall and Lee (1995) used immunocytochemistry to demonstrate masses of rod-staining neurofilaments in the axon stump by 12 hours post-lesion and which remained for 1-2 weeks post-axotomy. Based on these observations they concluded that neurofilament side-arm proteolysis as

the result of axotomy near the lesion site, allowed the access of antibodies to the rod domain. It was suggested that side-arm loss caused high packing density of neurofilaments within neurofilamentous masses near the lesion site. Basically similar findings were obtained by Pettus and Povlishock (1996) in a study of moderate/severe fluid-percussion brain injury in cats where HRP tracer (which is normally excluded by the intact axolemma) was administered intrathecally. Local alterations in axolemmal permeability to HRP were detected. The axolemmal permeability was consistently associated with distinct axonal cytoskeletal changes. Within 5 minutes of injury there was a significant decrease in interneurofilament spacing in injured axons which contained HRP. The decrease in spacing between neurofilaments in these axons was associated with a lack of neurofilament side-arm projections. The increase in density of neurofilaments, as measured by the number of neurofilaments per $0.025 \mu\text{m}^2$ of axoplasm, was associated with a decrease in microtubule density. More recently, neurofilament side-arm loss and compaction of neurofilaments has also been reported after impact acceleration in rats (Povlishock *et al.*, 1997).

However, increase in packing density of neurofilaments is not limited to traumatic brain injury. It also has been described in other pathologies such as Parkinson's and neurodegenerative diseases (Hill *et al.*, 1991; Trojanowski *et al.*, 1993). But in traumatic brain injury, it may be that an increase in neurofilament packing density concomitant with loss of neurofilament side-arms would result in local cytoskeletal collapse, perhaps associated with axonal shrinkage. All the ultrastructural changes observed in HRP-containing axons within 5 minutes of injury persisted unchanged up to 6 hours after traumatic brain injury (Pettus and Povlishock, 1996). Following the latter observations Pettus and Povlishock (1996) suggested that a change in

axolemmal permeability caused a rapid, persisting general cytoskeletal change, with local cytoskeletal collapse/alteration, probably related to local ionic dysregulation. They also hypothesised that an impairment of axonal transport as the result of cytoskeletal collapse/alteration might be associated with upstream axoplasmic swelling which ultimately leads to detachment. Pettus and Povlishock (1996) also observed that within the same microscopic fields other injured axons were present in which there was neurofilament misalignment rather than compaction 5 minutes after injury. This neurofilament misalignment also resulted in impairment of axoplasmic transport, leading to the formation of axonal swellings and finally, disconnection 3 hours after traumatic brain injury. Therefore two different pathological cytoskeletal responses were observed in two population of axons (i) HRP-containing axons with neurofilament compaction and microtubular loss and (ii) non-HRP-containing axons with neurofilament misalignment but without microtubular loss. Maxwell *et al.*, (1994a, b) also provided evidence for increased spacing or decreased density for both neurofilaments and microtubules in the axons of the guinea pig optic nerve 6 hours after stretch-injury. More recently, Jafari *et al.*, (1997) and the present work have provided evidence for a differential response between the smallest and larger axons after stretch-injury in guinea pig optic nerve - *vide infra*.

It is established that side-arms, the carboxy-terminal tail domains of NF-M and NF-H, are involved in interaction between neurofilaments (Nixon and Sihag, 1991; de Waegh *et al.*, 1992; Brady, 1993; Nixon *et al.*, 1994). Phosphorylation of the carboxy-terminal may stabilise the carboxy-terminal in an extended configuration (Sternberger and Sternberger, 1983; Hagestedt *et al.*, 1989) and increase the angle of the side-arm extension. This increases the distance of extension and results in an increased

interneurofilament spacing (Nixon and Sihag, 1991; de Waegh *et al.*, 1992; Brady, 1993; Hsieh *et al.*, 1994; Nixon *et al.*, 1994; Tu *et al.*, 1995). Alternatively, negative charges associated with phosphorylation sites on the side-arm increase the repellent forces that help to maintain the distance between neurofilaments (Nixon and Sihag, 1991; de Waegh *et al.*, 1992; Brady, 1993; Nixon *et al.*, 1994). Further, it has been shown that treatment of cultured neurons of rat dorsal root ganglia with okadaic acid, which inhibits protein phosphatase 1 and 2A, caused disruption of the neurofilament network by hyperphosphorylation of the amino-terminal of neurofilament subunits and resulted in disassembly of neurofilaments (Sacher *et al.*, 1992). As was described before, there is some evidence in mammalian nervous system for a mechanism by where neurofilament dephosphorylation leads rapidly to proteolysis by calcium-independent, endogenous proteinase (Goldstein *et al.*, 1987). It has been suggested that proteolysis of neurofilament side-arms, whether by increase in intra-axonal Ca^{2+} concentration and activation of μM calpain or by dephosphorylation of neurofilament side-arms and activity of calcium-independent endogenous proteinase would result in loss of interneurofilament side-arms repellent forces (Pettus *et al.*, 1994; Hall and Lee, 1995; Pettus and Povlishock, 1996). The resultant loss of neurofilament side-arms would decrease interneurofilament spacing and so increase their packing density.

II.7.b. Microtubules

Studies on the effect of nocodazole on microtubules in axons of cultured sympathetic neurons of rats (Baas and Black, 1990; Baas *et al.*, 1991, 1993) revealed that there were two different classes of microtubule polymers that showed different

sensitivity to nocodazole. These studies showed that half of the microtubule mass was drug-labile and had a half time of depolymerisation of approximately 3.5 minutes, while the other half was relatively drug-stable and depolymerised with a half time of approximately 130 minutes. Baas and Black (1990) found that the labile domain of microtubules was located at the plus end of the stable domain. Nocodazole inhibited microtubule assembling by binding to unassembled tubulin while at the same time disassembly continued to occur at the normal rate (Hoebeke *et al.*, 1976; Lee *et al.*, 1980). In spite of many studies that distinguish cold-stable and cold-labile microtubules in axonal extracts (Webb and Wilson, 1980; Brady *et al.*, 1984; Watson *et al.*, 1990), Baas *et al.*, (1994) found that all or nearly all microtubule polymers in all axons of cultured rat sympathetic neurons from the superior cervical ganglia including drug-labile polymers were cold-stable. Baas and Heidemann (1986) and Baas *et al.*, (1987) demonstrated the existence of both cold-stable and cold-labile microtubule polymers in the axon of cultured chick sensory neurons grown for less than 24 hours. A proportion of microtubules found in the developing axons in the mouse tectal plate was found to be cold-labile (Cohen, Binet and Meininger, 1987). Baas and colleagues (1994) suggested that practically all of the microtubule polymer in the brain is cold-stable, but microtubule cold stabilising factors are easily lost during invasive preparative procedures which leads to depolymerisation of some microtubules in cold. They also suggested that cold-stabilising factors consistently increase during development of neuronal and axonal growth up to a time when most or all of microtubules become cold-stable. However, since in the intact adult lumbosacral nerves of eviscerated toads, incubated at 0°C, Alvarez and Fadic (1992) detected a substantial microtubular depolymerisation, Baas and colleagues (1994) suggested that

the level of cold-stable and cold-labile microtubule polymer may depend on the species, type of neuron and development.

In *in vitro* experiments it has been established that calcium has an inhibitory effect on tubule polymerisation at a 10^{-5} M concentration, with a magnesium concentration 20 times greater than the determined free intracellular concentration (1 mM) (Olmsted and Borisy, 1975). However, these latter experimental parameters do not reflect normal, physiological concentrations of either calcium or magnesium. Under such conditions, millimolar levels of calcium cause a rapid depolymerization of microtubules (Olmsted and Borisy, 1975) being half-maximal at 1.0 mM and with total inhibition at a calcium concentration greater than 2.0 mM. Further, there is a difference in calcium sensitivity between crude extract and purified microtubular protein. In a crude extract only a few microtubules were left at 5×10^{-5} M calcium concentration, and no microtubules were observed at concentration of 5×10^{-4} M. However, in purified microtubular proteins extracts many microtubules were still present even a 1.0 mM concentration of calcium (Nishida and Sakai, 1977). Alternatively, it has been reported that in mammalian brain tissue depolymerisation of cold-labile microtubules occurred quickly under low concentrations, < 1.0 mM, of calcium (Weisenberg, 1972; Gaskin *et al.*, 1975; Olmsted and Borisy, 1975; Nishida and Sakai, 1977). More recently, Job *et al.*, (1981) reported that rat brain purified cold-stable microtubules assembled *in vitro* did not disassemble in either a calcium concentration of less than 5 mM or a high concentration of calmodulin, but quickly disassembled in the presence of both calcium and calmodulin at physiological concentrations. Further, the assembly of microtubules of bovine brain was totally inhibited after 5 minutes preincubation in experimental buffer with 0.50 mM Ca^{2+} containing either μM or mM calpain (Billger *et al.*, 1988).

Addition of μM calpain and 0.50 mM of Ca^{2+} to the steady state of assembly of microtubule proteins into microtubules caused a rapid disassembly of microtubules (Billger *et al.*, 1988). Inhibition of microtubule assembly was associated with complete degradation of high-molecular weight microtubule associated proteins 1 and 2 after a few minutes of incubation (Billger *et al.*, 1988). The above data indicated the current controversy in this area and the necessity to integrate and correlate *in vitro* data with levels of calcium, and other important monovalent and divalent ions, both in normal and injured axons. However, it is concluded that calcium has a greater and faster effect on either inhibition of assembly or disassembly of microtubules with a lower concentration in the presence of magnesium, calmodulin, μM and mM calpains than when it is used by itself at a higher concentration, such that disassembly of microtubules occurs rapidly in the presence of μM calpain, calmodulin and 0.50 mM calcium (*vide supra*).

Loss of axonal microtubules has been reported in various studies: for instance in large diameter axons of the rat optic nerve one hour after anoxic injury (Waxman *et al.*, 1992), 5 minutes to 3 hours after moderate/severe fluid-percussion brain injury in cats (Povlishock *et al.*, 1995) and 5 minutes to 6 hours in large axons in the same injury (Pettus and Povlishock, 1996), in nodes of Ranvier 15 minutes to 2 hours (Maxwell, 1996) and in the internode of larger axons up to 24 hours after stretch-injury (Maxwell, 1996; Jafari *et al.*, 1997; Maxwell and Graham, 1997) to the optic nerve of the guinea pig. Maxwell (1996) hypothesised that the activation of brain calpain as the result of influx of Ca^{2+} may be associated with the reduction in the number/density and proteolysis of microtubules followed by the presence of a

flocculent substructure in the node of Ranvier and internodal portion of the axon (Maxwell *et al.*, 1994a, b) after stretch-injury.

II.8. Pathological changes of the myelin sheath and its association with alteration in axonal calibre

Under pathological conditions the myelin sheath is often difficult to fix. It is sometimes difficult to distinguish whether a change in the myelin sheath is as the result of inadequate fixation or reflection of a pathological condition. In spite of these facts, some studies show pathological changes after the application of stretch or tensile strain to nerve fibres. Ochs and Jersild (1990) demonstrated that after mild-stretch (2-10 g) to dorsal and ventral roots of the cat and sciatic nerves of the rat, small intrusions formed in the internodes or near the constriction of beaded nerve fibres prepared by fast-freezing and freeze-substitution. The direct inward folding of the inner myelin lamellae formed myelin intrusions in or near the constrictions. Spheres formed from myelin lamellae consisted of major dense lines and intraperiod lines just like those found in the myelin sheath. Some intrusions were observed in unbeaded nerve fibres, detached from the myelin sheath, having a regular compact myelin lamellae with a surrounding axolemma. It was suggested by Ochs and Jersild (1990) that those intrusions which did not have an axolemmal covering and had variable disorganised and irregular shape of lamellae, were undergoing fragmentation and dissolution in the fibre.

In nerve fibres undergoing beading, splitting of lamellae and separation of the major dense lines may occur as the result of influx of cytoplasmic fluid Schwann cells from adjacent constriction (Ochs and Jersild 1990). In addition to lipids, water which

constitutes about 30-50% of myelin sheath's weight (LeBeaux and Willemot, 1975; Kirschner *et al.*, 1984), moved longitudinally from constriction to adjoining myelin sheath, forcing apart their component lamellar membranes and resulted in asymmetric localised separation of the lamellae: this was described as leafing (Ochs and Jersild, 1987).

Myelin sheath disruption also has been observed to occur in optic nerve fibres of guinea pigs after stretch-injury (Maxwell *et al.*, 1995). In this study 15 minutes after injury, focal disruption of the myelin sheath was associated with a loss in labelling for ecto-Ca-ATPase activity. The loss of ecto-Ca-ATPase activity and increased precipitation of pyroantimonate showed that the intra-axonal Ca^{2+} concentration was increased at sites of myelin sheath disruption. Therefore Maxwell *et al.*, (1995) suggested that perhaps the influx of Ca^{2+} occurred through the sites where the myelin sheath was disrupted.

Ochs and Jersild (1987) demonstrated that in association with contraction of the axon at the beading constriction, the circumference of the myelin sheath was greatly reduced as much as 1/3 to 1/5 of its normal size. Importantly, despite the reduction of myelin sheath diameter, its thickness did not change. The distance measured between major dense lines of myelin lamellae was 14 nm in both constricted and non-constricted regions. Ochs and Jersild (1987) suggested in order for this occur in the myelin sheath the lipid and most likely other components of the myelin lamellar membrane must move longitudinally from constrictions in the plane of lamellar membranes within 5-10 seconds.

II.9. Correlation with diffuse axonal injury in human head-injury

As a result of the great deal of effort in recent years to reveal the pathology of traumatic axonal injury in experimental laboratory settings in animals as a model for human DAI, it is now clear that (i) axons, except at the severest levels of injury are not sheared or torn at the time of injury but rather (ii) enter a so-called “pathological cascade” of events that result in compromised axonal transport, the formation of axonal swellings and secondary axotomy occurring at least several hours after injury. Studies using more sensitive techniques for the labelling and identification of damaged axons have been applied to cases of human head-injuries and have provided a better understanding of axonal pathology in diffuse axonal injury; and, perhaps most importantly, clearly demonstrated that the time course of axonal responses in head-injured humans is longer than that documented in animal models. Further, use of these more sensitive labelling techniques have allowed the development of the concept that TAI is far more widespread in humans than only occurring in cases of DAI. Axonal injury after mild head-injury in humans has been reported by Oppenheimer (1968) and after concussion by Blumberg *et al.*, (1994) in addition to the various grades of human diffuse axonal injury that have been documented by other investigators (Adams *et al.*, 1989; Grady *et al.*, 1993; Christman *et al.*, 1994; Gentleman *et al.*, 1995; McKenzie *et al.*, 1996).

Immunolabelling of the 68 kDa neurofilament subunit have been used to detect axonal swelling in humans after head injuries (Grady *et al.*, 1993; Christman *et al.*, 1994). More recently, and with a much greater sensitivity than earlier methods, immunocytochemical labelling for β -APP has been used to detect axonal damage or axonal swelling after head injuries in humans (Gentleman *et al.*, 1993, 1995; Sherriff *et*

al., 1994; Blumbergs, 1994, 1995; McKenzie *et al.*, 1996). By the use of this immunocytochemical technique axonal swellings first could be recognised as early as 1.75 hours (Blumbergs *et al.*, 1995) and 2 hours (McKenzie *et al.*, 1996). But only at 6 hours by use of immunolabelling for the 68 kDa neurofilament subunit (Grady *et al.*, 1993; Christman *et al.*, 1994). As survival time continued focal axonal swelling increased until axonal disconnection occurred 12 hours after injury (Grady *et al.*, 1993; Christman *et al.*, 1994). However, clearly disconnected reactive swellings were recognised until between 30 and 60 hours after injury (Christman *et al.*, 1994). At this survival time the reactive swellings contained a dense immunoreactive 68 kDa immunoreactive neurofilament subunit neurofilament core that sometimes was capped by a non-immunoreactive zone containing mainly mitochondria and smooth endoplasmic reticulum. The reactive axons were larger and elongated, consistent with the continued delivery of organelles by axoplasmic transport 88 hours after injury (Grady *et al.*, 1993; Christman *et al.*, 1994). Again, at this survival time the reactive axonal swellings contained a dense immunoreactive core of neurofilaments. But the majority of the reactive axons were encompassed by a significantly expanded non-immunoreactive zone (Christman *et al.*, 1994). However, perhaps as an indication of the heterogeneity in the process of reactive axonal change, some reactive axons exhibited a lesser expansion of the axis cylinder and thereby appeared similar to reactive swellings at an earlier stage of development (Christman *et al.*, 1994). The different survival times, 2-99 days after mild head injury (Blumbergs *et al.*, 1994) and 6-88 hours after severe traumatic head injury (Christman *et al.*, 1994), and the differential pathological changes strongly suggest that the situation is more complex than there being just a single post-traumatic time course leading to axonal

disconnection and provides support for the hypothesis that reactive axons show a spectrum of pathological events after injury (Povlishock, 1992; Pettus and Povlishock, 1996; Maxwell *et al.*, 1997b).

In animal models of traumatic brain injury, similar events were demonstrated by Yaghmai and Povlishock (1992). In this study, as the reactive swelling expanded, the immunoreactive neurofilament core began to be encompassed entirely by the developing nonimmunoreactive organelle-laden cap 2-6 hours after injury. But, as noted earlier (pages 44-45), the time course is much shorter in experimental animals than in human beings. The time course of axonal change increases from small animals to larger ones such that in rats and cats axonal disconnection occurs between 2 and 6 hours after injury (Erb and Povlishock, 1988; Povlishock, 1992), in miniature swine retraction balls (axonal bulbs) occurs 6 hours after lateral head acceleration (Ross *et al.*, 1994), but in humans axonal disconnection has not been observed until 12 hours post-injury (Grady, *et al.*, 1993; Christman *et al.*, 1994). Ultrastructural observation of intra-axonal events shows great similarities between findings in human head-injury and experimental animals ranging from cats to micropigs. Ultrastructural studies of humans 6 hours after head-injury showed that focal accumulation of immunoreactivity seen at the light microscopic level was associated with an expanded axis cylinder laden with 68-kD immunoreactive neurofilaments (Christman *et al.*, 1994). Neurofilament misalignment was occasionally associated with infolding of the axolemma (Christman *et al.*, 1994) comparable to early stages of axonal injury in animals. By 12 hours after injury some axons showed continuous neurofilament misalignment, pronounced immunoreactivity, vacuolisation, and occasional disconnection (Christman *et al.*, 1994). By 30-60 hours after injury accumulation of neurofilaments and organelles

caused further expansion of the axis cylinder and clearly disconnected reactive swellings were recognised. This enclosed a randomly organised neurofilamentous core encompassed partially by a cap of less densely aggregated organelles, which is expanded in longer survival time (Christman *et al.*, 1994). Based on the findings obtained from human traumatic brain injury, it is concluded that evidence obtained from animal models demonstrates that in both humans experiencing DAI and in animal models of TAI, 1. There is a strong indication that cytoskeletal changes could play an important role in the genesis of subsequent reactive axonal change (Christman *et al.*, 1994), 2. The changes may not be involved in great break down of cytoskeleton, but rather could be involved in reorganisation of both neurofilamentous and microtubular components of the axonal cytoskeleton, and 3. The changes are comparable with changes found in experimental animals but with a much longer time frame for humans.

III. The hypothesis to be tested in the present study

The purpose of the present study was to test the hypothesis that there is a spectrum of pathological changes (Povlishock and Christman, 1995; Maxwell *et al.*, 1997b) after stretch-injury (Gennarelli *et al.*, 1989; Maxwell *et al.*, 1990, 1991a, b, 1994a, b, c, 1995, 1997b; Maxwell, 1996; Maxwell and Graham, 1996, 1997; Jafari *et al.*, 1997). This hypothesis was tested on the internodal portion of stretch-injured nerve fibres of guinea pig optic nerves, an animal model of human diffuse axonal injury, at 4 hours survival time when axonal swellings are numerous (Povlishock *et al.*, 1983; Povlishock, 1992; Yaghmai and Povlishock, 1992; Maxwell *et al.*, 1997b). Gennarelli and colleagues have not provided precise electron microscopic quantitative morphometric data along the length of the optic nerve to support their statement that

the prechiasmatic portion of the nerve is the site where the most severe abnormalities occur after stretch-injury (Gennarelli *et al.*, 1989), now termed traumatic axonal injury. The only morphometric study that they have provided is the measurement of total axoplasmic area in the prechiasmatic and retrobulbar portion of control and stretch-injured optic nerves, where in comparison with controls, both portions showed a similar significant increase in axoplasmic area. Thus identification of the prechiasmatic portion of the optic nerves as the site of the occurrence of maximal abnormalities was mainly based on observation of the accumulation of HRP in this portion under the light microscope.

Therefore in the present study it was decided to re-examine the stretch-injured optic nerves to determine the site of maximal axonal damage based on electron microscopic studies. In order to do this and to test the above hypothesis the following morphological and morphometric analysis was carried out in different sizes of axons/fibres of control and stretch-injured optic nerves of guinea pig:

1. Measuring fibre parameters including:- axon diameter, fibre diameter and myelin sheath thickness.
2. Taking the proportion and the number of axons/fibres (axon + myelin sheath) falling within the bin sizes of $0.50\ \mu\text{m}$ of the internodal axon/fibre diameter along the length of optic nerves.
3. Taking the g-ratio, that is the ratio of axon diameter to fibre diameter.
4. Measuring the interneurofilament and intermicrotubule spacing.
5. Taking the density (that is the number of neurofilaments/microtubules per unit area of $0.0206\ \mu\text{m}^2$ of axoplasm) of neurofilaments and microtubules.

6. Counting the number of neurofilaments and microtubules in the axoplasm of each axon.
7. Counting the number of major dense lines of the myelin lamellae.
8. Providing the relationship between axon diameter and the number of myelin lamellae.
9. Providing the relationship between the number of neurofilaments/microtubules and axon calibre.
10. Morphological examination of longitudinal and transverse thin sections of the nerve fibres.

CHAPTER TWO

MATERIALS AND METHODS

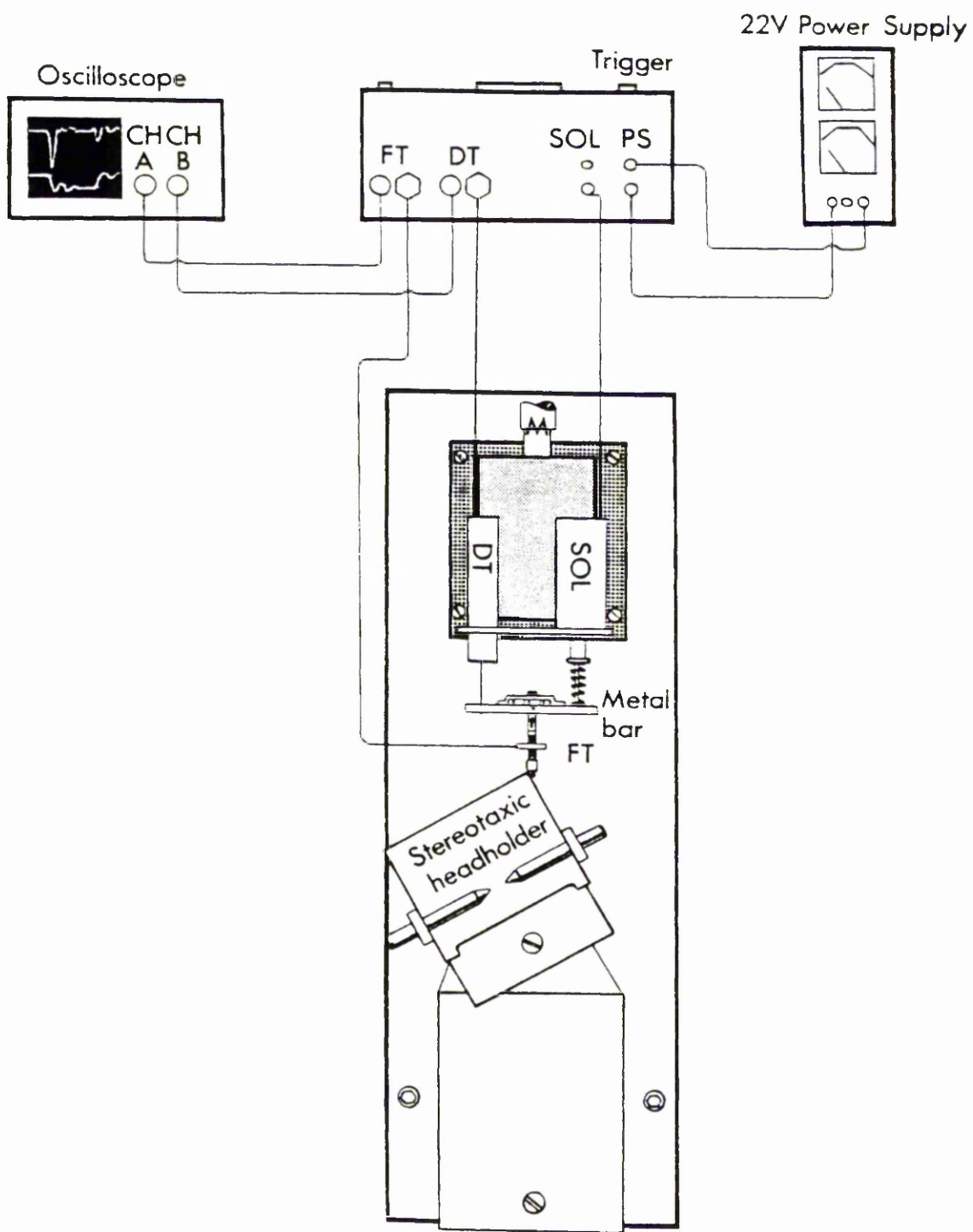
I. Model Preparation

Material was obtained from 6 adult, male albino Duncan Hartley guinea pigs (Harlan UK) (range 650-750 gm). Three animals were used as sham-operated controls and three were animals examined at 4 hours after stretch-injury to the right optic nerve. Animals were deeply anaesthetised with intramuscular ketamine (50 mg kg^{-1}) and xylazine (3 mg kg^{-1}). The right eyelids were infiltrated with 1% xylocaine containing epinephrine. The lids were retracted with 4/0 silk sutures and a lateral canthotomy was performed. With the aid of an operating microscope, a 360° opening of the conjunctiva was performed peripheral to the limbus, including the extraocular muscles. The globe was retracted temporally and the optic nerve was dissected free from any attachments but retaining its blood supply intact. A sling fashioned from sterile umbilical tape, moistened with isotonic saline, was placed over the globe and positioned firmly against the posterior pole. The sling was secured by tying sutures passed through the sling and tied at the front of the globe but ensuring that there was no contact with the cornea. In the 3 sham operated animals the sling was then removed.

II. Biomechanics and Stretch-Injury Apparatus

The stretch-injury apparatus (Gennarelli *et al.*, 1989) was custom-designed to deliver a reproducible and measurable amount of elongation or tensile strain to the optic nerve (Fig. 1). At the beginning of each experiment the stretch-injury apparatus was calibrated using a 100 g weight. The head of each of 3 anaesthetised experimental animals was placed into a stereotaxic headholder of the apparatus and secured by use of ear canal pins. The loose end of the umbilical tape sling was aligned towards a

Fig. 1. This stretch-injury apparatus is custom-designed to deliver a reproducible and measurable amount of elongation to the optic nerve. Displacement of the optic nerve occurs as a pulse generator activates the solenoid (SOL) piston to provide the force that is required. A force transducer (FT) measures the variations in force with respect to time. The displacement per time change is stored in a Tektronix 468 digital storage oscilloscope. (PS = Power source, DT = Displacement transducer, CH = Channel)



pulley on the injury apparatus before being tied about 10 mm in front of the eye. The pulley was connected to an inertially compensated force transducer. The headholder had three degrees of freedom of motion and was manoeuvred horizontally and vertically so that the pulley, sling and optic nerve were aligned along the long axis of the optic canal. The alignment had previously been established from dissection to be 30° laterally and 20° above the intraorbital line (Gennarelli *et al.*, 1989). Precise alignment was necessary to ensure that the line of force applied to the optic nerve was exactly axial to the optic canal in order to prevent bending of the nerve around the lip of the canal and thus reducing the risk of undesirable localised injury. To keep the initial state of the nerve as constant as possible, the same initial amount of force (30-40 g pre-load) was placed on each nerve such that there was no anatomical slack in the nerve. This was accomplished by advancing the transducer on a screw thread until the desired reading from the force transducer equal to the pre-load value was achieved. Displacement of the globe occurred as a pulse generator activated a solenoid piston to provide a force ranging between 150 and 180 g (plus the pre-load to give a total load of 180-220 g) over periods ranging from 19 to 21 ms (Gennarelli *et al.*, 1989). At this force level, there were no instances of optic nerve avulsion or of traumatic vascular injury which resulting from tearing of blood vessels in the optic nerve or retina. Throughout the operation the eye was moistened with isotonic saline and xylocaine at frequent intervals. A force transducer measured variations in force with respect to time, and a displacement transducer recorded the displacement-time history of the event. Both were recorded permanently either on a Tektronix 468 digital storage oscilloscope and the trace photographed using a Polaroid CR-9 Land camera; or on a polygraph pen recorder. All animals were maintained for 4 hours under light, halothane

(1% in oxygen) anaesthesia in order to minimise post-operative discomfort. Immediately after injury the sling was carefully removed and eyelids replaced over the globe. The area of the palpebral fissure was regularly moistened with isotonic saline and xylocaine to minimise drying of the cornea. All animal procedures were approved by the Home Office in the UK.

III. Fixation Procedures and Dissection

All animals were terminally anaesthetised with intraperitoneal barbiturate. After all respiratory movement had ceased, animals underwent thoracotomy for transcardiac perfusion. The vasculature was flushed with mammalian Ringer for about 1 minute followed by perfusion with 2.5% glutaraldehyde in 0.2M PIPES buffer (pH 7.6, 360 mOs) (Baur and Stacey, 1977) for 30 min. Animals were decapitated at least 30 minutes after perfusion fixation, and the whole head immersion fixed in the same fixative for 2 hours at 4°C. The brain, globes and optic nerves were then removed.

IV. Tissue Processing for Transmission Electron Microscopy

IV.a. Tissue collection and postfixation

Each right (experimental and sham control) and left (internal control for the quantity of fixation in control and experimental animals) nerve was cut into 3 segments, 4-6 mm in length, the length of guinea pig optic nerve being 12-20 mm (Gennarelli *et al.*, 1989), segment 1 adjacent to the globe, segment 2 the midportion and segment 3 the prechiasmatic portion of the nerve. Material was left in fixative for a further 36-48 hours at 4° C. The middle segment of the left optic nerve from each experimental and each control animal was used as an internal control for the quality of

fixation. Segments were washed in 2% sucrose in PIPES buffer three times each time for 20 minutes and postfixed in 1% osmium tetroxide in PIPES buffer for one hour. They were then quickly rinsed three times with distilled water.

IV.b. Dehydration and Araldite embedding

Specimens were dehydrated through 50%, 70%, 90%, and three changes in 100% concentrations of ethanol each of them for 30 minutes, and embedded in Araldite as follows.

Cleared in propylene oxide (2 changes)	20 minutes each
Propylene oxide/Araldite 753 mixtures (1:1)	over night
Propylene oxide/Araldite 753 mixtures (1:2)	12 hours
100% Araldite 753 (2 changes)	for a minimum of 12 hours each
Embedding in fresh Araldite at 60° C	24 hours

The recipe for Araldite resin used for the embedding medium was:

Araldite (resin)	12.5 g
DDSA (hardener)	12.5 g
DMP 30 (accelerator)	0.38 g
Dibutyl phthalate (plasticizer)	0.68 g

IV.c. Semithin sections and staining

The blocks containing the embedded optic nerve segments were mounted onto cured resin stubs with Rapid Araldite. The blocks were trimmed and cut with a Reichert-Jung Mod. 1140/Autocut. For light microscopical examination, transverse semithin sections were cut from one randomly selected end of each segment (3

segments of the right and the middle segment of the left optic nerve, a total of 4 segments in each control and experimental animal) until a complete transverse section was obtained. 10-15 transverse semithin sections at 0.50-1.00 μm were cut for purposes of orientation of the block. These sections were stretched, dried and adhered to glass slides on a hot plate. They were stained with Toluidine blue and viewed under the light microscope in order to determine an optimum orientation for the cutting of axonal transverse sections for transmission electron microscopy.

IV.d. Thin sections and staining

IV.di. Transverse thin sections

Ultrathin sections, 50-80 nm thick were cut on a Reichert-Jung Ultracut E Ultramicrotome using a Diatome 45° diamond knife. Gold and silver sections were stretched with chloroform vapour and collected on 300 mesh copper grids (Agar Aids, Berkhamstead, U.K.). Ten serial, transverse thin sections were cut from each segment of the right control (n = 3, 90 sections) optic nerve, the middle segment of the left optic nerve in control and experimental animals (n = 6, 60 sections) and each segment of the right stretch-injured optic nerves (n = 3, 90 sections) in experimental animals, a total of 240 sections. The middle segment of the left optic nerve in all animals was used as an internal control for the quality of fixation. All thin sections were stained with 12.5% methanolic uranyl acetate for 3 minutes, washed in distilled water for 1 minute and air dried. Then they were stained with lead citrate for 3 minutes (Donaghy *et al.*, 1988) followed by washing in distilled water for 1 minute and air drying in a dust free area. In order to achieve a representative sample (Williams, 1977) of transverse sections of the optic nerves, one random thin section from the 10 sections

from each segment of an optic nerve was selected from each animal, that is 4 sections from each animal. A total of 24 thin sections were examined in either a Philips 301 or Philips 100 TEM.

IV.dii. Longitudinal thin sections

After transverse thin sections had been cut and examined, 5 longitudinal semithin sections from each of the second and third segments of stretched-injured optic nerves, were cut and stained, as described above for transverse semithin sections, for light microscopical examination to achieve the optimum orientation of the block. 5 grids of serial longitudinal thin sections of these segments from all experimental animals, giving a total of 30 grids, were cut. All sections were stained as described earlier. Sections on the third grid in the series of sections were picked, and areas of particular interest in the preceding and following grids were examined.

Morphometric Studies

V.a. Calibration of Transmission Electron Microscope

The TEM was calibrated for $\times 3250$ and $\times 6750$ magnifications using a S104 line grating (2160 lines/mm). Calibration at high magnification was determined by using beef catalase crystals S124 (each line = 8.75 nm) [Agar Aids, Berkhamstead, UK] (Wrigley, 1968). The last micrograph in any cassette of micrographs was of a calibration graticule. The negatives of these graticules were also used in the Kontron vidas image analysis system as a calibration for measurement of fibre parameters, interneurofilament and intermicrotubule spacing and neurofilament and microtubule density per standardised area of axoplasm.

V.b. Fibre parameters measurements

To determine whether all fibres in the optic nerve respond in the same way to stretch-injury, the following variables were measured for each fibre:- fibre cross-sectional area, axonal cross-sectional area, fibre diameter (defined as the transverse diameter of the outer limits of the myelin sheath), axon diameter (defined as the transverse diameter of the axoplasm), myelin sheath thickness and the g-ratio (the ratio of axonal diameter to fibre diameter (Guy *et al.*, 1989)). Because fibres and the axons circumferences were irregular, it was difficult to measure their diameter precisely. Therefore to calculate their diameters, first the area of each was measured, then the diameter of a circle of that equivalent area was calculated and taken as either the fibre or axon diameter. Axons and nerve fibres (axon + myelin sheath) were grouped in 0.5 μm wide bins and analysed to determine whether there were changes in the number or proportion of the axon and nerve fibres within bins after stretch-injury.

V.bi. Sampling procedure

In order to measure fibre parameters the following sampling procedure was performed. The top left hand corner of each section examined was found. The first complete grid square covered by the section was used as the starting point for the gathering of data. Starting from this reference point the top left hand corner of each third successive grid square completely overlain by the section, (horizontally from left to the right) throughout the whole transverse section of the optic nerve was photographed at x 3,250 magnification. This usually resulted in 10 micrographs giving a sample of circa 700 nerve fibres in each transverse section of the optic nerve. All images collected from the segments of the control and four hour survival stretch-

injured optic nerves (n = 6), 17082 fibres, were recorded in a Kontron vidas image analysis system.

V.bii. Measurement methods

A macro program was written to measure the fibre parameters. A standard reference square for the purpose of alignment was drawn on a piece of paper. At the beginning of each run (the process that the computer took to measure fibre parameter or any other measurements stepwise from beginning to the end was called: run), this square was placed under the camera and its image superimposed on the image stored in the computer. In this way the camera position was exactly the same for each run. A micrograph of the line grating replica S 104 (2160 lines/mm) taken at the end of each cassette of micrographs at a magnification of x 3,250 was used as a scale (10 lines = 4.63 μm). The computer software used allowed direct measurement and assessment of axonal area in μm^2 . Each micrograph was placed under the camera, and the region of interest was stored in the computer and viewed on the vidas screen. The perimeter of each fibre (3-5 in fibres each run) was outlined using a digitising tablet. In each fibre, the myelin sheath was discriminated. In fibres in which the axolemma had a close relationship with the myelin sheath, the remaining part of the fibre (excluding the myelin sheath) was recognised as the axon by the computer. In the fibres where the axolemma had lost its close relationship with the myelin sheath there existed periaxonal space(s) (Maxwell *et al.*, 1995). In these fibres a line was drawn around the axolemma manually to make the axon recognisable by the computer. This procedure was carried out in each run until the profiles of all of the fibres (that is about 70) in a

field of each negative were then collected. At the end of each run, the fibre parameters and the g-ratio were measured.

V.c. Interneurofilament and intermicrotubule spacing measurements

V.ci. Sampling procedure

Results of the analysis of changes in axonal and fibre parameters listed above demonstrated a great increase in the number or proportion of the smallest axons (diameter of less than 0.50 μm) in optic nerves after stretch-injury. Gennarelli *et al.*, (1989) have also documented changes in the proportion of larger axons and fibres after stretch-injury. Therefore it was decided to start investigation of changes in two groups of axons, the smallest with a diameter of less than 0.50 μm and the larger with an axonal diameter of greater than 2.00 μm .

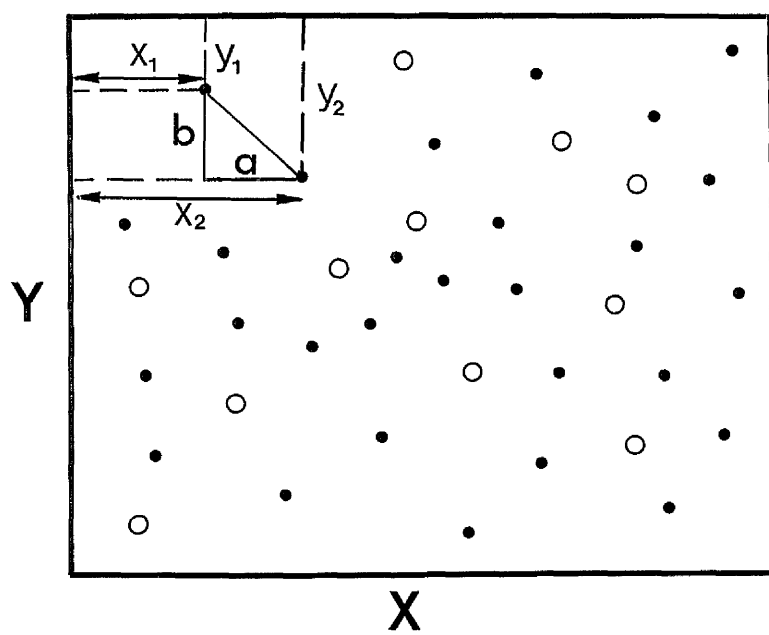
It was hypothesised that the increase in the number or proportion of axons after stretch-injury was as a consequence of reduction in the axonal calibre, and the axonal calibre may be reduced as a result of neurofilament compaction. Therefore in order to test the hypothesis that neurofilament compaction occurred after stretch-injury, an analysis of changes in the spacing of neurofilaments and microtubules in the smallest and the larger axons was carried out. The following procedure was performed. The top left hand corner of the transverse section of each segment of an optic nerve was found and used as an arbitrary starting point from which perpendicularly sectioned internodal portions of nerve fibres with axonal diameters of both less than 0.50 μm and greater than 2.00 μm were photographed at $\times 52,000$. A nerve fibre was selected as being transversely sectioned where the circularity factor was 0.8 or greater, and neurofilaments and microtubules were sectioned perpendicular to their longitudinal

axes. Axons within the bin sizes defined above were then regularly sampled as one axon in every third successive grid square (horizontally from left to the right) encountered throughout the whole transverse section of the optic nerve. This sampling procedure was used to generate 10 electron micrographs from each transverse section. In this way 20 axons (10 smallest and 10 larger ones) from each segment of control and injured optic nerves (in 24 transverse sections), a total of 480, were sampled.

V.cii. Measurement methods

Two macros were written for this measurement. The first was used to store images and the second was used to measure (i) the spacing between the neurofilaments (NFs) and (ii) the spacing between microtubules (MTs) from the previously stored images. First, the reference square was placed under the television camera to allow the accurate alignment of that camera. The micrograph of each nerve fibre was then placed under the camera. On the image of the axoplasm, first neurofilaments and then microtubules were digitised manually. The images of neurofilaments and microtubules were stored in the first macro. These images were used in the second macro to measure the spacing between neurofilaments and between microtubules as follows. Screen locations of all the NFs/MTs for elements x , y (x = distance from the left hand edge of the screen and y = distance from the top edge of the screen), were found. The individual co-ordinates of a particular NF/MT were compared arithmetically with the next nearest neighbouring NF/MT, the next NF/MT being identified by the allocation of grid positions, and implementing a concentric method for choosing the next to be measured. The a & b (fig. 2) distances were obtained by subtracting x_1 from x_2 which are the distances of first and second NFs/MTs respectively from the left edge of the

Fig. 2. This diagram represents a computer image of axoplamic cytoskeletal components [neurofilaments (dots) and microtubules (circles)]. The spacing between each neurofilament or microtubule and its nearest neighbour is measured by calculating the length of the hypotenuse of the right angle triangle with the sides of length (a) and (b).



screen ($a = x_2 - x_1$), and y_1 from y_2 being the distances of first and second NFs/MTs from the top edge of the screen ($b = y_2 - y_1$). The Pythagoras theorem ($hyp^2 = a^2 + b^2$) was used to calculate the distance between two NFs/MTs which is the hypotenuse of a right angle triangle with sides a and b . In this way the distances between a particular NF/MT and its 4 nearest neighbouring NFs/MTs were measured. The mean distances between one particular neurofilament or microtubule and its four neighbours was calculated. The results for all axons, 20 (10 in each group) from each segment in each control and in each experimental animal, were used to calculate the mean spacing either in the control or stretch-injured optic nerve in each animal. Mean of means and standard error of means were calculated from control and experimental animals.

VI. Determination of Changes in the Second Segment of Optic Nerve

The analysis of changes in the number or proportion of the smallest axons and the occurrence of fibres possessing periaxonal space(s) was greatest in the second segment (see results pages 102-104 and 111). Results from the spacing analysis demonstrated differential changes in the spacing of the components of the axonal cytoskeleton after stretch-injury between the smallest and the larger axons in the second segment of the optic nerve (see results pages 109-111). Therefore, it was decided to investigate changes further in the second segment of stretch-injured optic nerves.

VI.a. Interneurofilament and intermicrotubule spacing measurement

In addition to the micrographs of axons which previously were taken for spacing measurements in the second segment of stretched-injured optic nerve, 5 axons from each experimental animal a total 15 axons with periaxonal space(s) were

photographed. Thus the overall total number of axons analysed including all axons from segments of control optic nerves in control and experimental animals was 375.

VI.b. Calculating the density of neurofilaments and microtubules per unit area of axoplasm

Interim results provided evidence for differential changes in spacing between neurofilaments and between microtubules in the smallest and the larger axons. To determine whether changes in spacing occurred as a result of either changes in the density of neurofilaments and microtubules within a standardised area of axoplasm, or as a result of a change in their number, the study was extended to provide data for changes in the density of neurofilaments and microtubules and for the total number of neurofilaments and microtubules within the cross-sectional area of control and stretch-injured axons.

To determine the density of neurofilaments and microtubules within a standardised area of axoplasm, a macro was written in the vidas image analysis system. In order to obtain a uniform magnification throughout the whole sample, a circle drawn on a piece of paper, stored as an image in the computer, was used to adjust the position of the camera. At the magnification for electron micrographs used in this study, a circle with an area of $0.0206 \mu\text{m}^2$ was drawn in the computer. In order to draw a circle, the length of radius should be given to the computer. The radius was the only character used by the computer to draw a circle with a certain size. Since the scale in the computer was in pixels, and the length of radius must be given in this scale, it was difficult to get a round number for the area of the circle after converting it into microns. Therefore, the circle that was drawn with the area of $0.0206 \mu\text{m}^2$, was as near as possible to a circle

with an area of $0.02 \mu\text{m}^2$. The error here is therefore $- 0.0006 \mu\text{m}^2$. A micrograph of beef catalase crystals S 124 taken at x 52,000 magnification was used as a scale to allow the calculation of the area of the circle.

Micrographs that were taken at x 52,000 magnification for spacing measurement from all segments of control optic nerves (*vide supra*) and the second segment of stretch-injured axons were used to calculate the density of neurofilaments and microtubules within a standardised unit area (the above circle) of axoplasm. Each micrograph was placed under the camera. The circle was superimposed on the image at a randomly selected area of the axoplasm. Neurofilaments and then microtubules were digitised within the circle. In the smallest axons with a diameter of $0.50 \mu\text{m}$ or less, because of the small size of the axon, 2 unit areas per axon, giving a total of 360 (20 from each segment of the right and middle segment of the left control optic nerves in each control and experimental animals, and the middle segment of stretch-injured optic nerve in each experimental animal, where $n = 3$) unit areas were collected. In larger axons with a diameter greater than $2.00 \mu\text{m}$, because of the fact that the distribution of neurofilaments and microtubules was not uniform throughout the axoplasm (See figs. 7a, b and 8a, b), in order to have a good representative sample, 4 unit areas per axon, giving a total of 720 (40 from each segment of the right and left control optic nerves in each control and experimental animals (*vide supra*) and the middle segment of the stretch-injured optic nerve in each experimental animal where, $n = 3$) unit areas were collected. In larger axons with periaxonal space(s) 4 unit areas per axon a total of 60 (20 from each experimental animal where, $n = 3$) unit areas were collected. A total of 1140 unit areas over the whole sample of control and experimental animals were therefore analysed.

Data were collected in the image analysis system. The mean density of neurofilaments or microtubules per $0.0206 \mu\text{m}^2$ of axoplasm was calculated from 10 axons (in each group of the smallest and the larger axons), and 5 axons with periaxonal space(s) in the second segment of stretch-injured nerves in each experimental animal, and 10 axons (in each group of the smallest and the larger ones) from each segment of the right and middle segment of the left optic nerves in control animals, and the middle segment of the left optic nerves in experimental animals. Mean of means and standard error of means were calculated from each control and each experimental animal.

VI.c. Counting the number of neurofilaments and microtubules

To determine whether there was a change in the number of neurofilaments and microtubules in the stretch-injured axons, the total number of each in control and stretch-injured axons in which neurofilaments and microtubules were perpendicularly sectioned were counted. The criteria chosen for microtubules and neurofilaments to be counted were that microtubules in any axon appeared as discrete circles of 20-26 nm in diameter and neurofilaments as discrete electron dense dots (10 nm in diameter) respectively. In the smallest axons the micrographs that were taken for spacing measurement at x 52,000 magnification, were also used to count the number of neurofilaments and microtubules. In these axons the counting was done directly from the micrographs by placing them on an X-ray box. For larger axons micrographs of related axons that were taken at x 11,700 magnification were placed under a WILD M5 dissecting microscope with drawing tube attachment at x75 magnification. Neurofilaments and microtubules were counted in the whole cross-sectional area of

the axoplasm, after marking them on a paper. The mean number of microtubules and neurofilaments in 10 axons within each control and each experimental group in each segment, except for those axons with a periaxonal space (5 axons only), in each animal were counted. The results from all animals ($n = 6$) from which the mean and standard error of the number of neurofilaments and microtubules in 150 control and 30 of the smallest stretch-injured axons, and 150 control and 30 larger axons (in addition to 15 axons in the larger fibres in which periaxonal space was present) were calculated.

VII. Determination of Changes in the Intermediate Bin Size Fibres with an Axonal Diameter Ranging from 0.51 to 2.00 μm

The results obtained from the smallest and the larger axons showed remarkable changes in the axonal cytoskeleton after stretch-injury (see results chapters 3 and 4). In order to test the hypothesis that axons in the middle size range (with an axonal diameter ranging from 0.51 to 2.00 μm) also showed changes in the organisation of the cytoskeleton after stretch-injury, it was decided to investigate whether these axons respond in the same way or differently than the smallest and the larger axons to stretch-injury. The experiments, described above, were repeated for axons between 0.51 and 2.00 μm diameter. The sampling procedures and measurement methods were the same as described above, except that, the axons which were sampled for spacing measurement at high magnification, also were photographed at low magnification to measure their fibre parameters.

VII.a. Relationship between number of myelin lamellae and axon diameter

It has been demonstrated that the number of myelin lamellae is correlated with the axonal diameter (Hilderbrand and Hahn, 1978), and that myelin sheath thickness and the periodicity of major dense lines remain the same in constricted and non constricted/or expanded regions of beaded nerve fibres (Ochs and Jersild, 1987). Thus, in order to distinguish whether a fibre was shrunken, swollen or had a normal size, and then categorise it in a proper axonal bin size, after stretch-injury, the major dense lines of myelin lamellae in each bin size in both control and experimental animals were counted.

To plot a graph, from control fibres, to show the relationship between the axon diameter and the number of myelin lamellae, and also to be used as a reference for sampling the proper axonal size in the stretch-injured axons, additional data, besides the data from the fibres in the intermediate bin size (as will be described in the following paragraphs), was necessary. To generate these data, 10 axons from each bin size of 0.00-0.50, 2.01-2.50 and 2.51-3.00 μm from each control optic nerve giving a total of 90 axons was collected.

VII.b. Fibre parameters and interneurofilament and intermicrotubule spacing measurements

To measure fibre parameters and spacing between neurofilaments and between microtubules, 10 axons from each bin size (0.51-1.00, 1.01-1.50, 1.51-2.00 μm), a total 30 from the middle segment of each control and each stretch-injured optic nerve in each animal (60 axons in each control and in each experimental animals, where $n = 3$) giving a total of 360 axons, were photographed at $\times 6750$ and $\times 52000$

magnifications respectively. The number of major dense lines of myelin lamellae in each fibre was counted (See chapter 5 and table 10).

VII.c. Calculating neurofilament and microtubule density per unit area of axoplasm and counting their number

The axons collected to measure spacing between neurofilaments and between microtubules were also used to calculate their density per standard unit area ($0.0206 \mu\text{m}^2$). The following procedure was performed to collect unit areas from the axons. For the axons within the bin size of $0.51\text{--}1.00 \mu\text{m}$, 2 unit areas in each axon, giving a total of 240 (20 in each control and in each stretch-injured optic nerves, a total of 40 in each control and in each experimental animals, where $n = 3$), for the axons falling within the bin sizes of $1.01\text{--}1.50 \mu\text{m}$ and $1.51\text{--}2.00 \mu\text{m}$, 4 unit areas in each axon, giving a total of 960 (80 in each control and each stretch-injured optic nerves, a total of 160 in each control and in each experimental animals, where $n = 3$) unit areas were collected. A total of 1200 unit areas over the whole sample of control and stretch-injured axons were collected.

To count the number of neurofilaments and microtubules over the entire transverse cross-section of the axons, the following procedure was carried out. In the axons within the bin size of $0.51\text{--}1.00 \mu\text{m}$, the same micrographs that were collected for spacing measurement also were used for counting (*vide supra*). In these axons the counting was done directly from micrographs by the aid of an X-ray box. In the axons with a diameter greater than $1.00 \mu\text{m}$ the dissecting microscope was used as described before.

The mean for each of the above measurements was calculated from 10 axons in each bin size from each optic nerve in each animal, and the mean of means and standard error of means were calculated from control and experimental animals.

VIII. Statistical Analysis

VIII.a. Significant changes in three segments of optic nerves

The significance of changes in the number of the smallest axons within the bin sizes of (0.00-0.50 μm), (0.51-1.00 μm) and the fibres within the bin size of 0.51-1.00, 1.51-2.00 and 2.51-3.00 μm in control and stretch-injured optic nerves were determined by means of the χ^2 test. Analysis of variance (ANOVA) was used to analyse any possible differences in the spacing between neurofilaments and between microtubules in the smallest axons (less than 0.50 μm in diameter) and the larger axons (greater than 2.00 μm in diameter) in all segments of control optic nerves. The significant of differences in the spacing of neurofilaments and microtubules between the smallest and the larger axons in the control optic nerves, and between each segment of stretched-injured optic nerves and controls, was determined by the Student's 't' test.

VIII.b. Significant changes in second segment of optic nerves

Changes in the fibre parameters and axonal cytoskeleton were marked (i) within two subgroups of nerve fibres, those with an axonal diameter of less than 0.50 μm and those with an axonal diameter greater than 2.00 μm (ii) in the intermediate bin size fibres with axonal diameter ranging from 0.51 to 2.00 μm . Analysis for statistical significance of changes in the fibre and cytoskeleton within the two groups of the

smallest and the larger fibres after stretch-injury was carried out in the following ways. The significance changes in spacing, density per unit area of axoplasm, number of neurofilaments and microtubules and the number of the myelin lamellae in the smallest fibres (with an axonal diameter of less than 0.50 μm) was determined by means of the Student's 't' test. Axons (greater than 2.0 μm in diameter) of the larger nerve fibres demonstrated two different changes after stretch-injury. The significance of changes in the g-ratio between the smallest control fibres (axon diameter < 0.50 μm), the larger control fibres (axon diameter > 2.00 μm), and the smallest stretch-injured fibres (Axon diameter < 0.50 μm), and the larger fibres with axonal diameter > 2.00 μm either with intramyelinic spaces or with periaxonal space(s) was determined by means of the Student's 't' test. The significance of changes in axonal diameter between control larger fibres and fibres with a periaxonal space(s), in the spacing, density per unit area, number of the neurofilaments and microtubules between each group of the stretch-injured and control larger axons was determined by the Student's 't' test.

VIII.c. Significant changes in the intermediate bin size axons

The significant of changes in spacing, density and number of neurofilaments and microtubules between each of bin size of 0.51-1.00, 1.01-1.50, 1.51-2.00 μm in control and stretch-injured axons was determined by the Student's 't' test.

RESULTS

CHAPTERS THREE TO FIVE

CHAPTER THREE

THE SITE OF MAXIMAL AXONAL DAMAGE IN THE THREE SEGMENTS OF STRETCH-INJURED OPTIC NERVES

I. The Number or Proportion of Axons and Fibres Within 0.50 μm Bins in the Control and Stretch-Injured Optic Nerve

I.a. Control optic nerves

Application of the χ^2 test showed that there were no significant differences in the number of axons/fibres falling within 0.50 μm wide bins of internodal axonal and fibre diameter between 0.00 μm and 4.00 μm in the 3 segments of the right sham optic nerves in control and the middle segment of the left optic nerves in both control and experimental animals (internal controls) (tables 1 and 2).

Since there was no significant difference between the segments of right sham controls and left internal controls (*vide supra*), the total number of axons or fibres combined from each segment of right sham control optic nerves in each control animals was compared with the segments from the stretch-injured right optic nerves in experimental animals.

I.b. Stretch-injured optic nerves

I.bi. Axons

Comparison of the results for all of the axons falling within 0.50 μm wide bins of the internodal axonal diameter between 0.00 μm and 3.00 μm in the segments of optic nerves in the 3 control and 3 experimental animals demonstrated a two (205.9%), five (506.3%) and one and half (158.3%) fold increase in the number or the proportion of axons with a diameter of less than 0.50 μm in the first, second and third segments of stretch-injured optic nerves (table 1). Comparison of changes in the number of these axons from the control, using the χ^2 test, showed that the differences in the probability

were highly significant in all three segments ($p < 0.001$) (table 1). But (according to χ^2 values) the difference was greatest in the second segment of the stretch-injured optic nerves (table 1). There was also a decrease by 10.5% in the number of the axons within the bin size 0.51-1.00 μm in the second segment of stretch-injured optic nerves. Application of the χ^2 test showed that this decrease in the number of axons was significant ($p < 0.05$) (table 1). These novel data were the first evidence that the greatest degree of axonal change occurred in the second rather than the third or perichiasmatic segment of the stretch-injured optic nerves, contrary to the finding of Gennarelli *et al.*, (1989). There was not any significant change in the number of axons in the remainder of the axonal bin sizes (intermediate and larger axons) in all three segments (table 1).

Table 1

The total number (NO) and proportion (%) of axons falling within 0.50 μm wide bins in 3 segments (S) of control (Right sham controls) and the stretch-injured optic nerves and middle segment of left optic nerves in control and experimental animals (Internal control)

								Internal controls (middle segment of left optic nerve)			
Bin size (μm)	S	Right sham controls		Right experimentals		χ^2 test		Control animals		Experimental animals	
		NO	%	NO	%	χ^2 value	p value	NO	%	NO	%
0.0-0.50	1	34	1.62	104	4.81	34	$p < 0.001$				
	2	32	1.52	194	9.49	120.86	$p < 0.001$	31	1.47	35	1.67
	3	36	1.67	93	4.03	21.28	$p < 0.001$				
0.51-1.0	1	1522	72.48	1574	72.77	0.01	n.s.				
	2	1536	72.83	1375	67.30	4.55	$p < 0.05$	1542	73.32	1507	71.83
	3	1575	72.98	1715	74.31	0.25	n.s.				
1.01-1.5	1	443	21.09	404	18.68	3.19	n.s.				
	2	448	21.24	398	19.48	1.62	n.s.	444	21.11	460	21.92
	3	455	21.08	432	18.72	3.16	n.s.				
1.51-2.0	1	74	3.52	57	2.63	2.79	n.s.				
	2	69	3.27	54	2.64	0.84	n.s.	65	3.1	72	3.43
	3	68	3.15	53	2.30	2.96	n.s.				
2.01-2.5	1	23	1.09	20	0.92	0.37	n.s.				
	2	21	0.99	16	0.78	0.72	n.s.	18	0.85	20	0.95
	3	22	1.02	15	0.65	1.73	n.s.				
2.51-3.0	1	4	0.19	4	0.18	0.00	n.s.				
	2	3	0.14	6	0.29	0.31	n.s.	3	0.14	4	0.19
	3	2	0.09	0.00	0.00	0.50	n.s.				

I.bii. Fibres

The number of fibres with a diameter falling within the bin size of 0.51-1.00 μm in segments one and three was increased by 23.4% and 24.1% respectively (table 2). The increase in the number of fibres in these two groups of bin sizes from control values, determined by the χ^2 test, was significant ($p < 0.001$ and $p < 0.01$ respectively) (table 2). On the other hand there was a decrease in the number of fibres with a diameter falling within the bin sizes of 1.51-2.00 μm and 2.50-3.00 μm by 19.4%, 16.0% and

50.0%, 52.6% in the first and third segments of stretch-injured optic nerves respectively (table 2). The χ^2 test demonstrated that these differences were significant (see table 2). The differences in the number of fibres in the second segment falling within all bin sizes and the rest of fibres, in the first and third segments, falling within the bin sizes of 1.01-1.50, 2.01-2.50, 3.01-3.50 and 3.51-4.00 μm were not statistically significant (table 2).

Table 2

The total number (NO) and proportion (%) of fibres falling within 0.50 μm wide bins in 3 segments (S) of control (Right sham controls) and the stretch-injured optic nerves and middle segment of left optic nerves in control and experimental animals (Internal control).

								Internal controls (middle segment of left optic nerve)			
Bin size (μm)	S	Right sham controls		Right experimentals		χ^2 test		Control animals		Experimental animals	
		NO	%	NO	%	χ^2 value	P value	NO	%	NO	%
0.51-1.0	1	727	34.62	897	41.47	13.14	p < 0.001				
	2	714	33.85	716	35.05	0.4	n.s.	715	34	691	32.94
	3	754	34.93	936	40.55	9.27	p < 0.01				
1.01-1.5	1	938	44.67	927	42.86	0.77	n.s.				
	2	945	44.81	850	41.60	2.5	n.s.	931	44.27	944	44.99
	3	935	43.33	997	43.2	0.005	n.s.				
1.51-2.0	1	320	15.24	258	11.93	8.71	p < 0.01				
	2	327	15.5	354	17.32	2.12	n.s.	336	15.98	340	16.2
	3	342	15.84	287	12.43	9.15	p < 0.01				
2.01-2.5	1	67	3.19	53	2.45	2.13	n.s.				
	2	71	3.37	74	3.62	0.25	n.s.	69	3.28	68	3.24
	3	72	3.34	61	2.64	1.93	n.s.				
2.51-3.0	1	34	1.62	17	0.78	6.35	p < 0.05				
	2	35	1.66	34	1.66	0.00	n.s.	36	1.71	37	1.76
	3	38	1.76	18	0.78	8.65	p < 0.01				
3.01-3.5	1	12	0.55	10	0.46	0.18	n.s.				
	2	14	0.66	10	0.48	0.66	n.s.	13	0.62	14	0.67
	3	13	0.60	9	0.39	0.02	n.s.				
3.51-4.0	1	2	0.09	1	0.05	0.25	n.s.				
	2	3	0.14	5	0.24	0.12	n.s.	3	0.14	4	0.19
	3	4	0.18	0.00	0.00	1.00	n.s.				

II. The Spacing Between Neurofilaments and Between Microtubules

Throughout the Length of the Optic Nerve

In order to test the hypotheses that axonal cytoskeletal responses to stretch-injury, were either diffuse or focal, changes in the spacing between (i) one neurofilament and its closest neighbouring four neurofilaments, and (ii) one microtubule and its closest

neighbouring four microtubules were analysed in the internodal portion of the nerve fibres in the segments of control and stretch-injured axons of guinea pig optic nerves. The analysis of changes were, initially, done in the smallest axons (diameter less than 0.50 μm), since there was a significant increase in the number of these axons after stretch-injury (*vide supra*), and larger axons with axonal diameter greater than 2.00 μm .

II.a. The spacing between neurofilaments and between microtubules in the smallest and larger control axons

There were no significant differences in the spacing between neurofilaments in the smallest axons ($f = 0.68$, $df = 4 \times 10$, n.s.), and larger axons ($f = 1.39$, $df = 4 \times 10$, n.s.) in the three segments of the right sham control, the middle segment of the left control optic nerves in control and experimental animals (internal control). Neither was there any difference in the spacing between microtubules in the smallest axons ($f = 0.61$, $df = 4 \times 10$, n.s.) and larger axons ($f = 0.14$, $df = 4 \times 10$, n.s.) (tables 3 and 4).

Table 3

Mean spacing (nm) (\pm SEM) between neurofilaments and between microtubules in the smallest axon with a diameter of less than 0.5 μm in the segments of control optic nerves

	Right controls			Internal controls (middle segment of left optic nerve)	
	Segment 1	Segment 2	Segment 3	Control animals	Experimental animals
Neurofilaments	122.75 \pm 3.40	134.18 \pm 12.3	118.41 \pm 5.31	121.78 \pm 7.45	122.89 \pm 3.90
Microtubules	100.63 \pm 6.93	93.84 \pm 7.57	97.02 \pm 2.59	104.75 \pm 4.58	102.00 \pm 4.3

Table 4

Mean spacing (nm) (\pm SEM) between neurofilaments and between microtubules in larger axon with a diameter $> 2.00 \mu\text{m}$ in the segments of control optic nerves

	Right controls			Internal controls (middle segment of left optic nerve)	
	Segment 1	Segment 2	Segment 3	Control animals	Experimental animals
Neurofilaments	70.04 \pm 2.17	78.29 \pm 4.36	73.24 \pm 3.16	73.85 \pm 0.55	75.51 \pm 0.51
Microtubules	168.12 \pm 11.00	170.00 \pm 6.95	174.22 \pm 4.73	170.78 \pm 3.20	167.40 \pm 7.00

Since the results demonstrated no difference in spacing between either microtubules or neurofilaments (i) along the whole length of the right optic nerve in the control axons and (ii) in the middle segment of the left optic nerve in control and experimental animals, the data from all of these segments was combined to give the overall mean for analysis of changes in the middle segment of stretch-injured optic nerves in experimental animals.

The results obtained from all segments of the control optic nerves stimulated the hypothesis that there were differences in the neurofilament and microtubule spacing between the smallest and the larger axons in the guinea pig optic nerve. Testing of this hypothesis demonstrated that the mean spacing between neurofilaments in the smallest axons (diameter of less than $0.50 \mu\text{m}$) (124.00 ± 2.70) was significantly higher than the mean spacing between neurofilaments in the larger axons (diameter greater than $2.00 \mu\text{m}$) (74.19 ± 1.4) by 67.1% ($t = 16.63$, $df = 5$, $p < 0.001$). But the opposite result was gained in respect of spacing between microtubules in the smallest and larger axons. The mean microtubule spacing in the smallest axons (99.65 ± 1.9) was

significantly lower than the mean microtubule spacing in the larger axons (170.10 ± 1.2) by 41.4% ($t = 31.23$, $df = 6$, $p < 0.001$).

II.b. Neurofilament and microtubule spacing in stretch-injured axons

II.bi. Smallest axons

Analysis of systematically selected transverse sections from all three segments of stretch-injured nerves demonstrated that in the smallest axons, less than $0.50 \mu\text{m}$ in diameter, there was an increase in neurofilament spacing by 29.8% in the first segment. This was not statistically significant from control (table 5) ($t = 2.51$, $df = 2$, n.s.). There was, however, a significant change in the spacing between neurofilaments in the middle and prechiasmatic segments of these axons. Indeed there were different changes in the two segments. In the second or middle segment, there was a significant decrease by 53.4%, in neurofilament spacing ($t = 12.13$, $df = 3$, $p < 0.002$), but a significant increase, by 55.6%, in the prechiasmatic portion or third segment ($t = 4.90$, $df = 2$, $p < 0.05$). However, the change in neurofilament spacing in the second segment was statistically more significant ($p < 0.002$) than the change in the third segment ($p < 0.05$). The decrease in spacing between neurofilaments in the smallest axons in the second segment resulted from the fact that neurofilaments became very close to each other and were termed compacted (meaning that neurofilaments were densely packed). This compaction of neurofilaments in the smallest axons, occurred only in the second segment of the stretch-injured optic nerve. The small increase in microtubular spacing in the stretch-injured axons was not statistically significant from control values in segment one (table 5) ($t = 1.08$, $df = 2$, n.s.), two ($t = 1.32$, $df = 2$, n.s.) or three ($t = 2.32$, $df = 4$, n.s.).

Table 5

Mean spacing (nm) and \pm SEM between neurofilaments and between microtubules in the smallest axons with axonal diameter less than 0.50 μm

	Control axons	Experimental axons segment 1	Experimental axons segment 2	Experimental axons segment 3
Neurofilaments	124.00 \pm 2.70	160.90 \pm 14	57.84 \pm 4.80	193.00 \pm 14
Change from control (%)		+29.76	- 53.35	+ 55.64
Microtubules	99.65 \pm 1.90	105.74 \pm 5.3	109.9 \pm 7.5	104.09 \pm 0.14
Change from control (%)		+ 6.11	+ 10.29	+ 4.45

II.bii. Larger axons

In larger axons (diameter greater than 2.00 μm) there was an increase in spacing between neurofilaments and between microtubules throughout the entire length of stretch-injured larger axons (table 6).

Table 6

Mean spacing (nm) and \pm SEM between neurofilaments and between microtubules in the larger axons with axonal diameter $> 2.00 \mu\text{m}$

	Control axons	Experimental axons segment 1	Experimental axons segment 2	Experimental axons segment 3
Neurofilaments	74.19 \pm 1.40	100.98 \pm 5.4	99.96 \pm 4.1	101.18 \pm 3.8
Change from control (%)		+ 36.11	+ 34.73	+ 36.38
Microtubules	170.10 \pm 1.20	249.0 \pm 14	267.42 \pm 4.1	250.92 \pm 5.2
Change from control (%)		+ 46.38	+ 57.21	+ 47.51

Statistical analysis showed that the 36.1%, 34.7% and 36.4% increased spacing between neurofilaments were significantly different in the first ($t = 4.78$, $df = 2$, $p <$

0.05), second ($t = 5.92$, $df = 2$, $p < 0.05$) and the third segments ($t = 6.75$, $df = 2$, $p < 0.05$) from control values (table 6). There was also a statistically significant increase in the spacing between microtubules in the first ($t = 5.58$, $df = 2$, $p < 0.05$), second ($t = 22.59$, $df = 2$, $p < 0.01$) and third segments ($t = 15.01$, $df = 2$, $p < 0.01$) in the same axons, by 46.4%, 57.2% and 47.5% respectively. But, as statistical analysis showed above, significant change in the neurofilament and in the microtubule spacing in the first segment was not as highly significant as in the second and third segments. It is noteworthy, however, that the increased spacing between microtubules was greatest in the second part of the nerve (table 6).

III. Comparison of the Proportion of Larger Fibres with Periaxonal Space(s) Throughout the Length of the Stretch-Injured Optic Nerves

Further evidence which allowed testing of the hypothesis that the second segment of the optic nerve was that portion of the length of the nerve where greatest axonal damage occurred after stretch-injury was provided by calculating the percentage of fibres within which periaxonal space(s) occurred (Maxwell *et al.*, 1995) (see next chapter). Periaxonal space(s) occurred in 1.3% of fibres in segment two, but only 0.1% and 0.4% in segments one and three respectively.

CHAPTER FOUR

MORPHOLOGICAL AND MORPHOMETRIC ANALYSIS IN THE SMALL AND LARGER AXONS OF THE SECOND SEGMENT OF STRETCH-INJURED OPTIC NERVES

The preliminary results in chapter three provided evidence that the greatest degree of axonal and fibre damage in the smallest fibres (with an axonal diameter of less than 0.50 μm) and larger fibres (with an axonal diameter greater than 2.00 μm) occurred in the second segment of optic nerves after stretch-injury. Therefore it was decided to investigate further the morphological and morphometric changes in the internodal portion of the smallest and larger fibres in the second segment of optic nerves in guinea pigs.

I. Analysis of Changes in the Smallest Fibres with an Axonal Diameter of less than 0.50 μm

I.1. Morphological analysis of transverse sections

I.1.a. Control fibres

In these fibres the myelin sheath was intact and myelin lamellae were uniformly arranged. The profile of the axolemma was smooth and located adjacent to the myelin sheath. Neurofilaments were spaced widely and there was a high number of microtubules throughout the axoplasm (fig. 3).

I.1.b. Stretch-injured fibres

In the stretch-injured fibres, the myelin lamellae were disorganised and in some parts in the myelin sheath were separated from each other (Maxwell *et al.*, 1995; Jafari *et al.*, 1997) (figs. 4, 5). In most of these fibres, the axon was shrunken, and the profile of the axon was irregular and there were foldings in the axolemma (figs. 4, 5). There was loss of the close relationship between the axolemma and the myelin sheath such that the axon had an irregular transverse profile (figs. 4, 5). The distance between

neurofilaments was much reduced from the value in control axons. Neurofilaments were compacted and grouped together to form a central core in the axoplasm (fig. 4) (Jafari *et al.*, 1997). There was a great loss in the number of microtubules in some axons (fig. 4), but in most axons the number of microtubules appeared to be close to the normal values (fig. 6).

Fig. 3. A transverse thin section from a control nerve fibre with an axonal diameter of less than 050 μm . This shows an uniform disposition of the myelin sheath, a smooth profile of the axolemma and the close relation of the latter with the myelin sheath. Neurofilaments (arrow) are widely spaced and there is a high number of microtubules (arrowhead). x 153 000.

Fig. 4. A transverse thin section of a stretch-injured nerve fibre with axonal diameter less than 0.50 μm . There is some degree of disruption of the myelin sheath. The axolemma has an irregular profile and the close relationship with the myelin sheath (arrows) has been lost. There is a reduction in the calibre of this axon. There is almost complete absence of microtubules in the axoplasm of this axon. The neurofilaments are closely packed and grouped within the centre of the axoplasm. x 153 400.

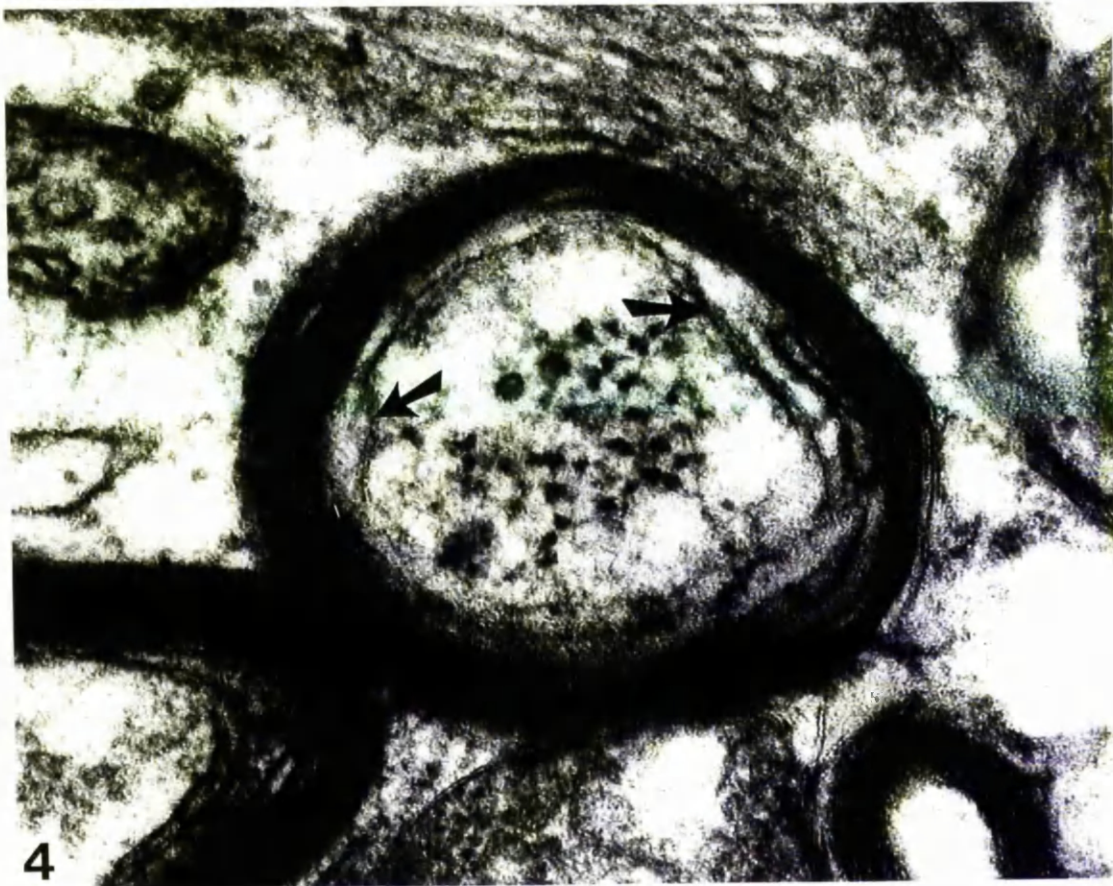
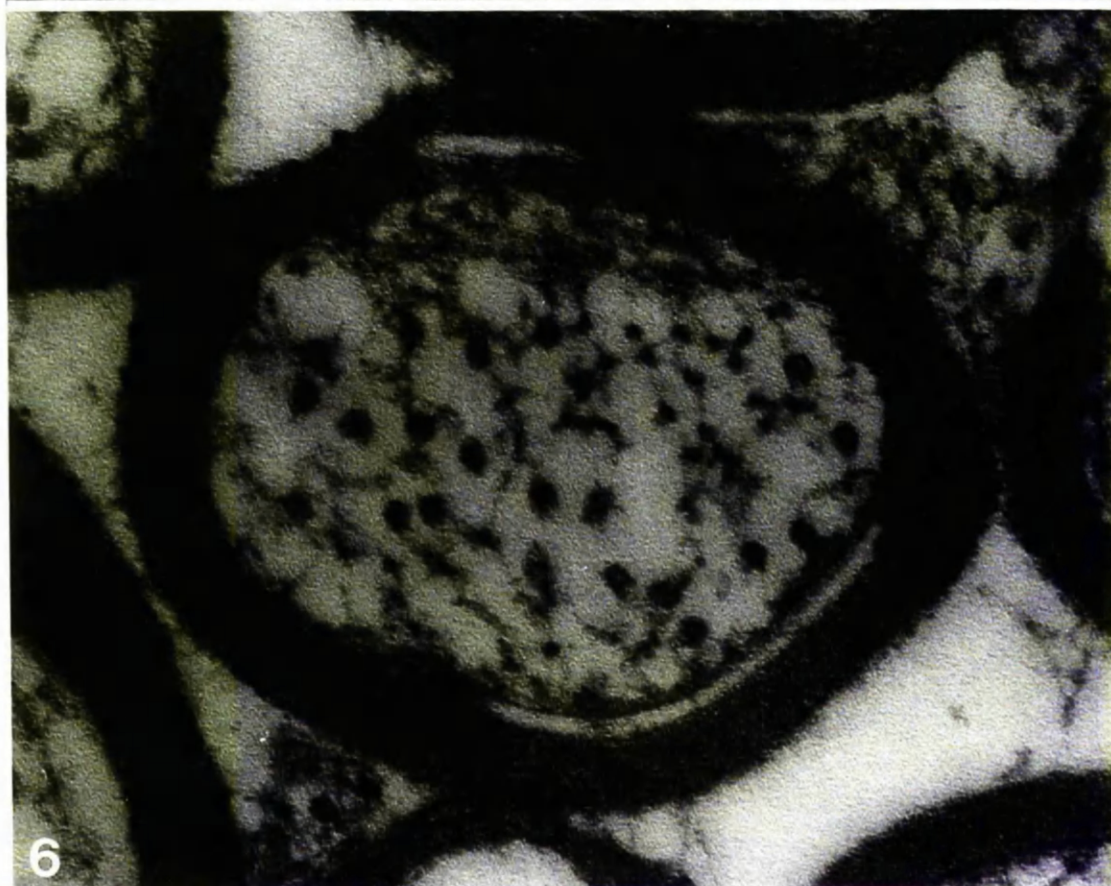
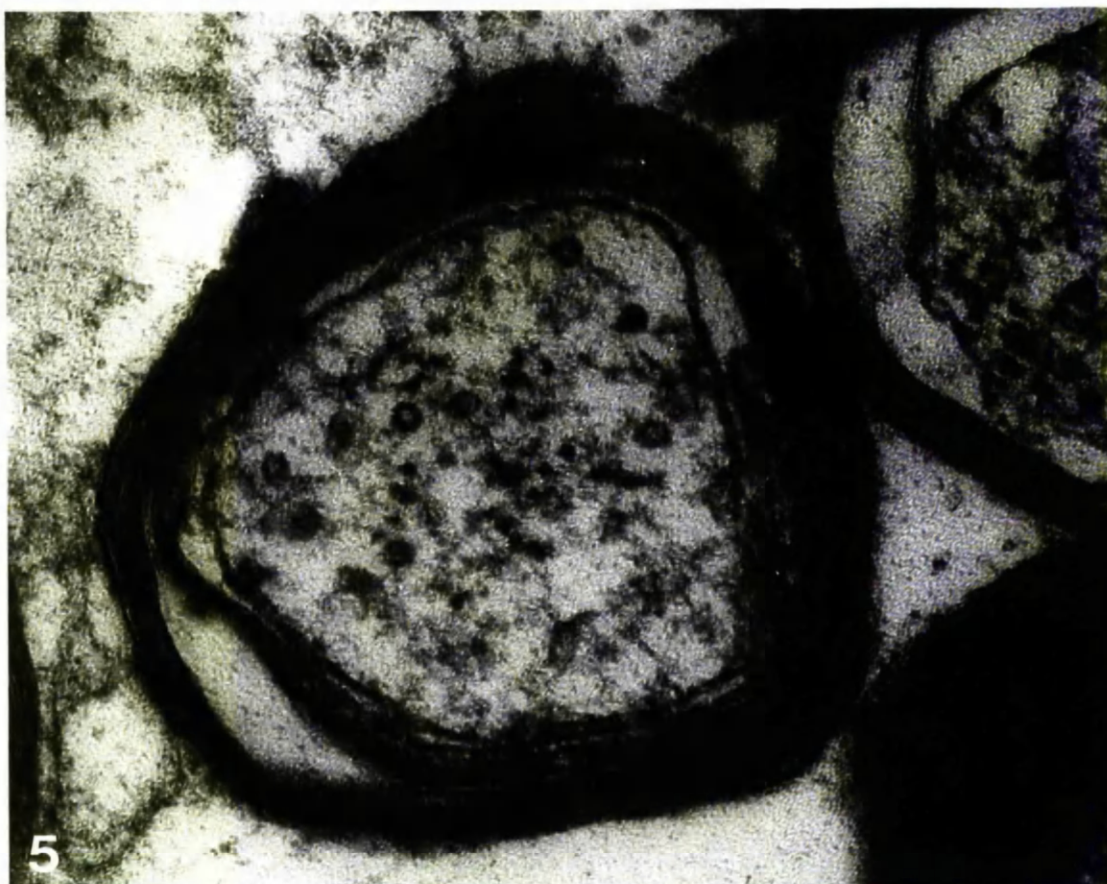


Fig. 5. Another example of a stretch-injured nerve fibre (diameter < 0.50 μm) which shows that neurofilaments are compacted and centrally grouped in the axoplasm even though a higher number of microtubules are present (compare with figure 4). x 150 000.

Fig. 6. In this small stretch-injured nerve fibre (axon diameter < 0.50 μm), there is a lesser degree of myelin sheath disruption and axolemmal irregularity. Although neurofilaments are compacted and they are not now grouped together in the centre of the axon. Also, there is a high number of microtubules in this axon. x 160 100.



I.2. Morphometric analysis of transverse sections

I.2.a. Changes in the g-ratio and the number of myelin lamellae

The g-ratio in the smallest fibres (axonal diameter $< 0.50 \mu\text{m}$) was reduced from 0.71 ± 0.01 in the smallest control fibres to 0.61 ± 0.02 in the stretch-injured fibres. That is a 14.1% reduction from the control values. This reduction was statistically significant ($t = 5.10$, $df = 3$, $p < 0.02$).

The number of myelin lamellae was increased from 4.93 ± 0.23 (mean \pm SEM) in control axons to 5.47 ± 0.42 (mean \pm SEM) (a percentage change of 10.95) in the stretch-injured axons. However the increase in the number of the myelin lamellae was not statistically significant ($t = 1.11$, $df = 3$, n.s.).

I.2.b. Changes in the spacing of neurofilaments and microtubules and their density per unit area of the axoplasm

Quantitative analysis of components of the axonal cytoskeleton in the smallest axons showed that where there was a significant reduction, by 53.4%, in spacing ($p < 0.002$). The density of neurofilaments per $0.0206 \mu\text{m}^2$ of axoplasm was increased significantly, by 254.4% ($t = 8.40$, $df = 2$, $p < 0.02$). But there was not a significant change in either the spacing of microtubules ($t = 1.51$, $df = 3$, n.s.), or their density per unit area of the axoplasm ($t = 3.97$, $df = 2$, n.s.) (tables 7 and 8; pages 120 and 121) (figs. 5 and 6).

I.2.c. Changes in the total number of neurofilaments and microtubules

Counts of the means of the total number of neurofilaments and microtubules in cross-sections of 150 control and 30 stretch-injured the smallest axons from 3 control and 3 experimental animals provided the data given in table 9 (page 122).

After stretch-injury, there was a significant increase ($t = 4.58$, $df = 3$, $p < 0.02$), by a factor of 99.9% (table 9; page 122), in the number of neurofilaments in the smallest axons. Therefore the increase in neurofilament density per unit area of axoplasm was correlated with both compaction and an increase in the number of neurofilaments (tables 7, 8 and 9; pages 121 and 122).

The 15.6% decrease in the number of microtubules was not statistically significant ($t = 0.75$, $df = 3$, n.s.) (table 9; page 122).

Table 7

Mean spacing (nm) and \pm SEM between neurofilaments and microtubules and their nearest 4 neighbours (combined means from 10 axons from each segment of optic nerve in each animal in each group, $n = 3$, and from 5 axons in which a periaxonal space was present)

	Control (smallest axons) < 0.50 μm	Experimental (smallest axons) < 0.50 μm	Control (larger axons) > 2.00 μm	Experimental (larger axons) > 2.00 μm	Experimental axons with periaxonal space(s)
Neurofilaments	124.00 \pm 2.70	57.84 \pm 4.80	74.19 \pm 1.40	99.96 \pm 4.1	57.20 \pm 1.20
Change from control (%)		- 53.35		+ 34.73	- 22.90
Microtubules	99.65 \pm 1.90	109.90 \pm 7.50	170.10 \pm 1.20	267.42 \pm 4.1	214.10 \pm 9.5
Change from control (%)		+ 10.29		+ 57.21	+ 25.87

Table 8

Mean density (\pm SEM) of microtubules and neurofilaments/ $0.0206 \mu\text{m}^2$ of axoplasm (combined means from 10 axons (5 with a periaxonal space) from each segment of optic nerve in each control and experimental animal ($n=3$, in each group))

	Control (smallest axons) < $0.50 \mu\text{m}$	Experimental (smallest axons) < $0.50 \mu\text{m}$	Control (larger axons) > $2.00 \mu\text{m}$	Experimental (larger axons) > $2.00 \mu\text{m}$	Experimental axons with periaxonal space(s)
Neurofilaments	2.52 ± 0.10	8.93 ± 0.76	5.51 ± 0.18	2.26 ± 0.11	10.26 ± 0.55
Change from control (%)		+ 254.36		- 58.98	+ 86.20
Microtubules	3.71 ± 0.05	2.73 ± 0.24	1.08 ± 0.05	0.47 ± 0.03	0.51 ± 0.02
Change from control (%)		- 26.41		- 56.48	- 52.77

Table 9

Mean number (\pm SEM) of neurofilaments and microtubules in cross-sections of axons in each control and experimental group (mean calculated from 10 smallest and 10 larger axons within each segment of optic nerve in each animal, a total sample of 360 axons, and 5 axons with a periaxonal space, from each experimental animal; $n=3$)

	Control (smallest axons) < $0.5 \mu\text{m}$	Experimental (smallest axons) < $0.5 \mu\text{m}$	Control (larger axons) > $2.0 \mu\text{m}$	Experimental (larger axons) > $2.0 \mu\text{m}$	Experimental axons with periaxonal space(s)
Neurofilaments	7.4 ± 1.3	14.8 ± 1.0	246.5 ± 9.0	179.9 ± 4.1	281.9 ± 8.2
Change from control (%)		+ 99.9		- 27.0	+ 14.4
Microtubules	11.4 ± 2.1	9.6 ± 1.1	74.6 ± 5.5	33.3 ± 1.9	20.7 ± 0.3
Change from control (%)		- 15.6		- 55.4	- 72.2

II. Analysis of Changes in the Larger Fibres with an Axonal Diameter

Greater than 2.00 μm

II.1. Morphological analysis of transverse sections

II.1.a. Control fibres

In the larger control fibres, the myelin lamellae were regularly disposed. The axolemma had a close relationship with the myelin sheath and the axon had a smooth transverse profile. There was a high number of neurofilaments and microtubules. Neurofilaments and microtubules were closely spaced (fig. 7a, b) (table 7; page 120).

II.1.b. Stretch-injured fibres

Larger fibres 4 hours after stretch-injury fell into two subclasses. Either those fibres with intramyelinic spaces (fig. 8a, b) or those fibres with a periaxonal space(s) (Jafari *et al.*, 1997) (figs. 9a, b and 10a, b).

II.1.bi. Nerve fibres with intramyelinic spaces

In nerve fibres with intramyelinic spaces, myelin lamellae were separated from each other to form intramyelinic spaces (Maxwell *et al.*, 1995), and myelin intrusions occurred (Ochs and Jersild, 1987 and 1990; Jafari *et al.*, 1997). The axolemma provided an irregular profile in transverse sections of axons but foci of separation of the axolemma from the myelin sheath to form periaxonal space(s) (Maxwell *et al.*, 1995; Jafari *et al.*, 1997) did not occur in these fibres. There was an increased spacing between neurofilaments and between microtubules. In comparison with control axons (fig. 7a, b) the loss in the number of microtubules in these axons was remarkable (fig. 8a, b).

Fig. 7. (a and b) A thin transverse section of a larger control nerve fibre (axonal diameter > 2.00 μm). (a) At low magnification the myelin sheath lamellae are regularly disposed, the axolemma has a smooth profile and is closely associated with the myelin sheath. (b) A detail of the axoplasm of the same fibre at a higher magnification to show the high number of both neurofilaments (arrows) and microtubules (arrowheads). These have a regular spacing of control values within the axoplasm. (a) x 34 600, (b) x 112 700.

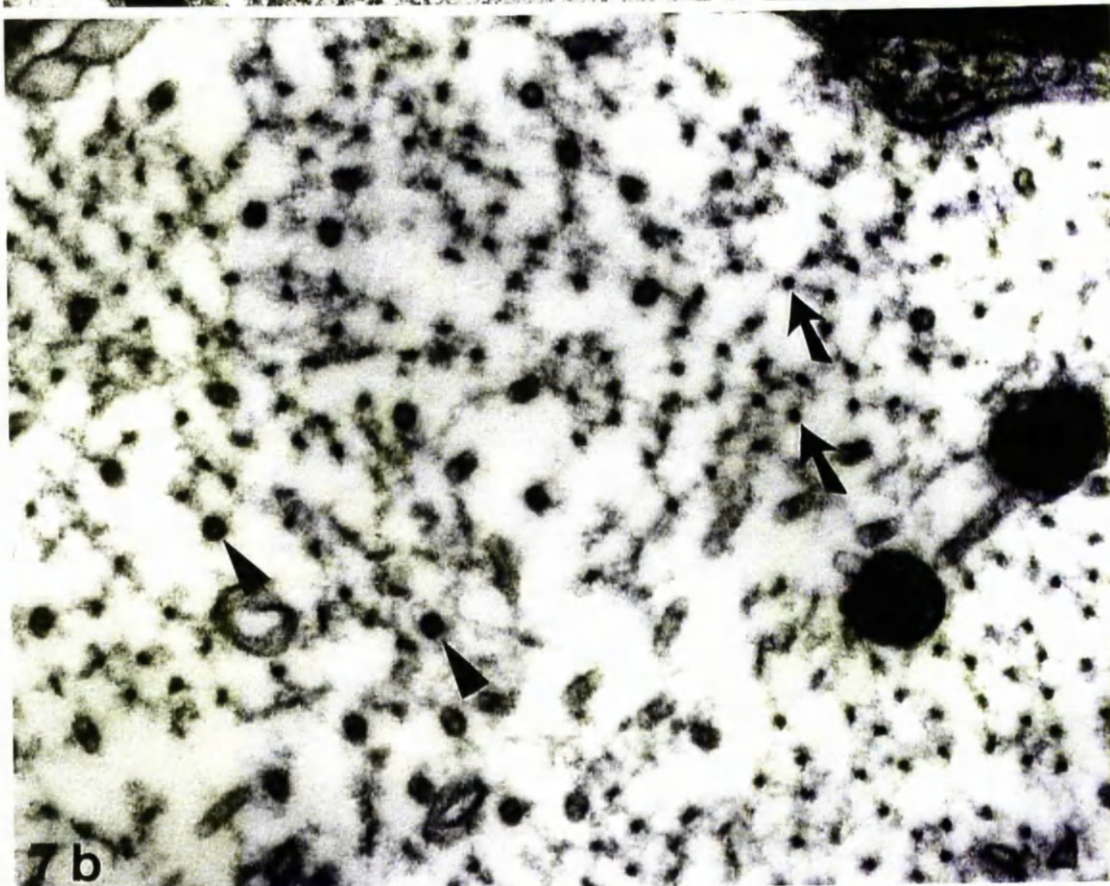
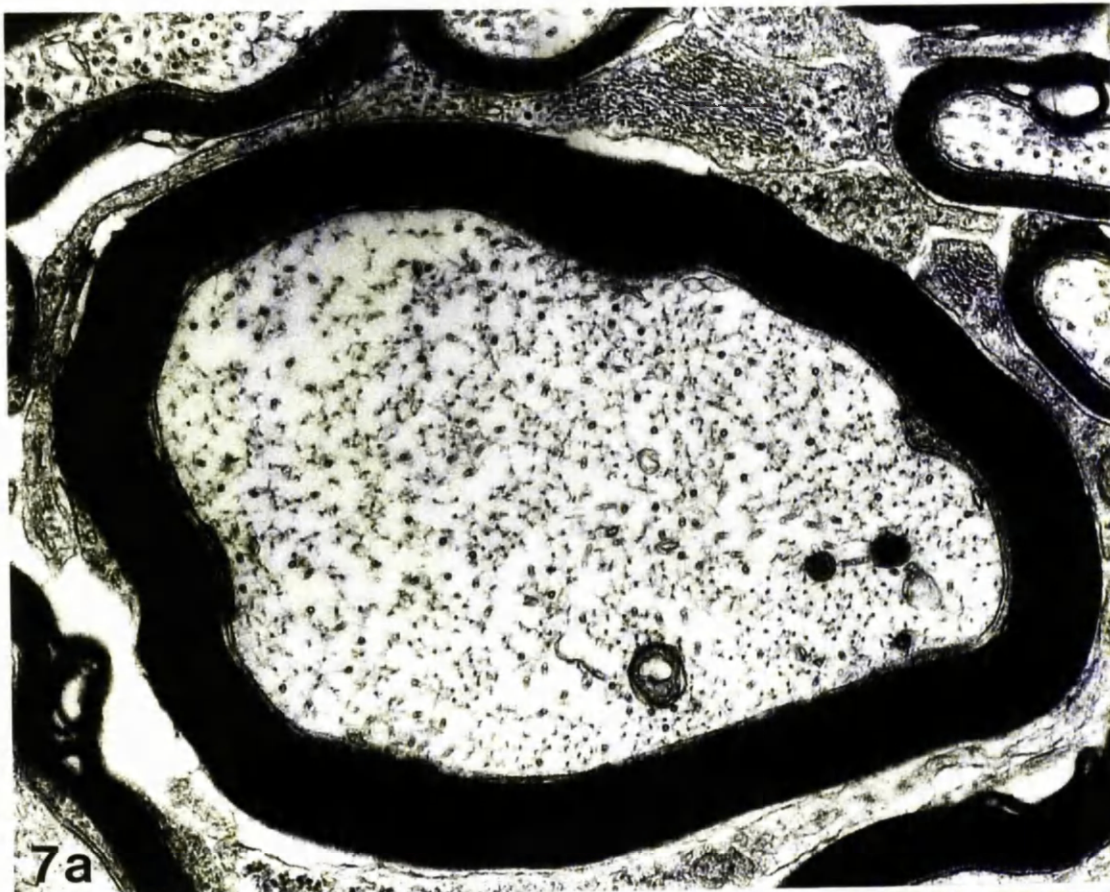
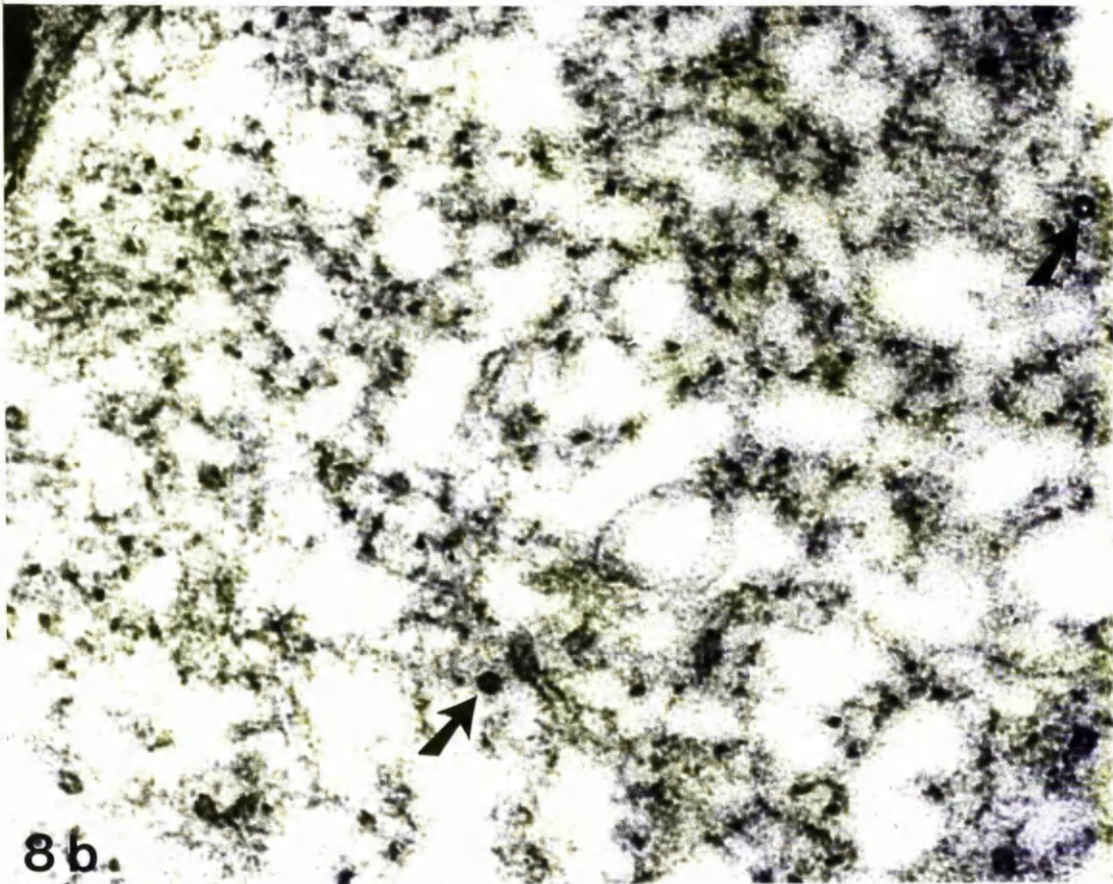
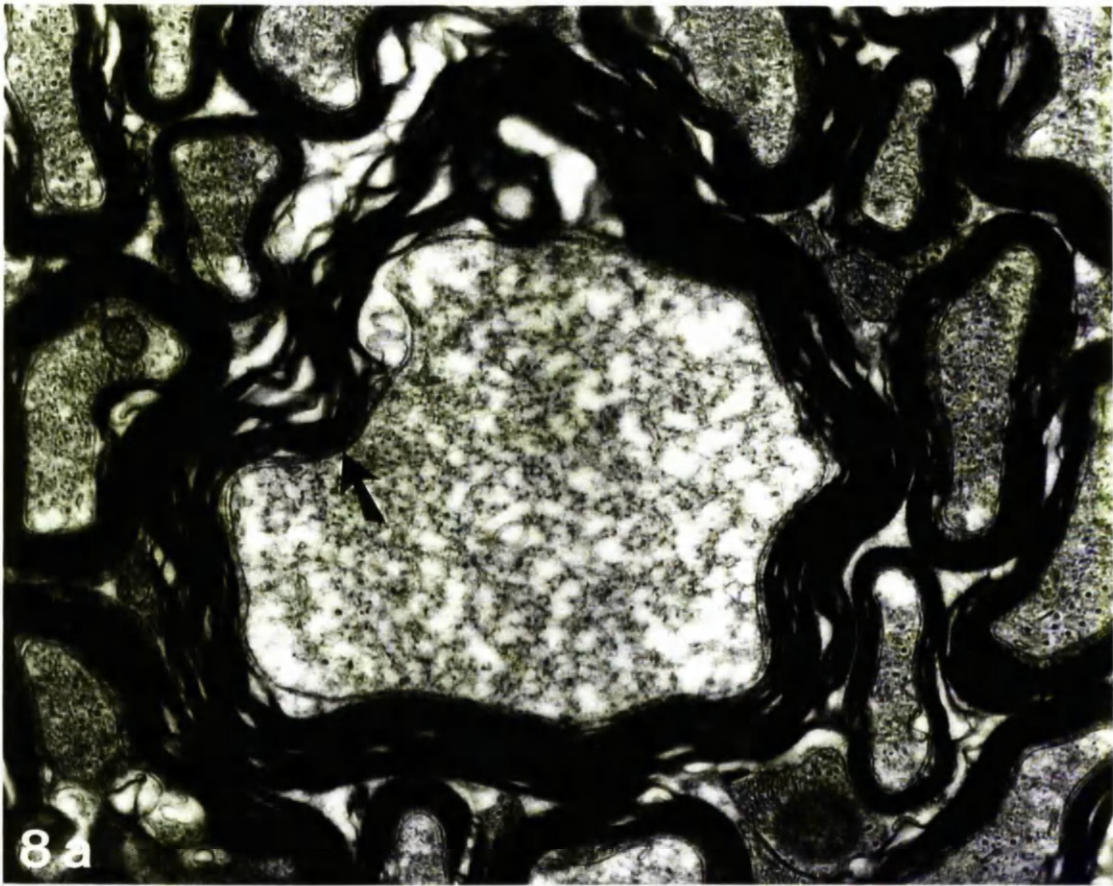


Fig. 8. (a and b) A transverse thin section of a larger nerve fibre (axon diameter > 2.00 μm) after stretch-injury. (a) There are sites of separation of myelin lamellae forming intramyelinic spaces, and myelin intrusions are present (arrow). There is separation of the axolemma from the internal aspect of the myelin sheath. The components of the axonal cytoskeleton are not regularly spaced. (b) A detail of part of the axoplasm at higher magnification. This shows an almost complete loss of microtubules (two of them are indicated by arrows) and an increased spacing between neurofilaments. (a) x 24 450, (b) x 97 100.



II.1.bii. Nerve fibres with periaxonal space(s)

In fibres in which periaxonal space(s) occurred (Maxwell *et al.*, 1995; Jafari *et al.*, 1997) (figs. 9a, b and 10a, b), the close relationship of the axolemma with the myelin sheath was lost. The axon was greatly shrunken and separated from the surrounding myelin sheath. The periaxonal space in most cases contained a flocculent material, and some membranous profiles (fig. 9a, b). In some fibres the periaxonal space was empty (fig. 10a, b) (Jafari *et al.*, 1997).

In the axoplasm neurofilaments were compacted, and in some axons microtubules were either grouped together in one part of the axon, or scattered in another part (fig 10a, b). Over all in comparison with control axons (fig. 7a, b), there was a marked loss in the number of microtubules in these injured axons (figs. 9a, b and 10a, b).

It was observed that in some fibres, a small area of the axonal cross-section contained neurofilaments, microtubules and some vesicles and was limited by a small portion of the axolemma (fig. 11a, b). The axolemma had a C-shaped profile at this region. The remainder of the cross section lacked any axonal recognisable cytoskeleton but rather the axoplasm was replaced by flocculent material. Some parts of the axolemma appeared to be broken. It is suggested that the part of the axon that contained recognisable cytoskeleton might be in the processes of being separated from the rest of the axon such that two lips of the axolemma and related glial processes fuse together to result in an enlarged periaxonal space.

Fig. 9. (a and b) A transverse thin section of a larger ($> 2.0\ \mu\text{m}$) nerve fibre after stretch-injury demonstrating (a) that there is a reduced calibre of the axon and the occurrence of a grossly enlarged periaxonal space. This periaxonal space contains flocculent material and some membranous profiles (arrow). (b) There is a very low number of microtubules, and neurofilaments are compacted. (a) x 26 600, (b) x 50 900.

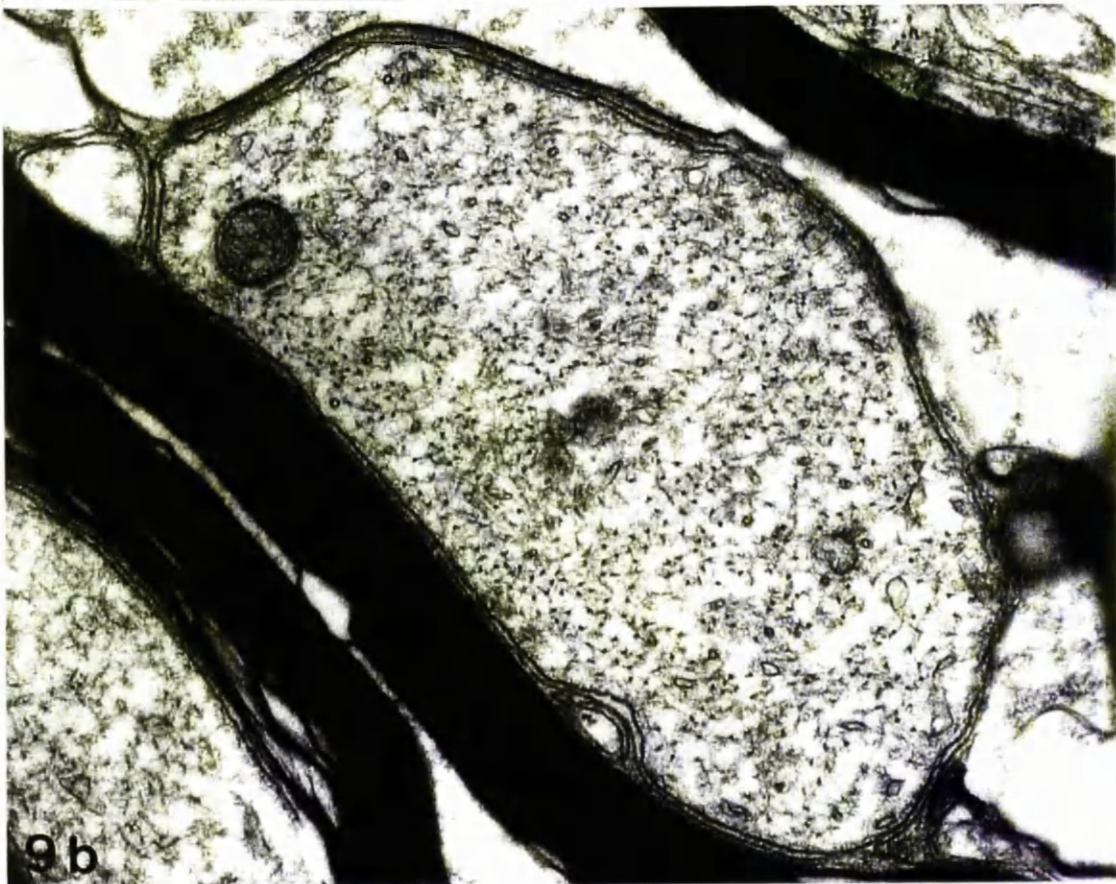
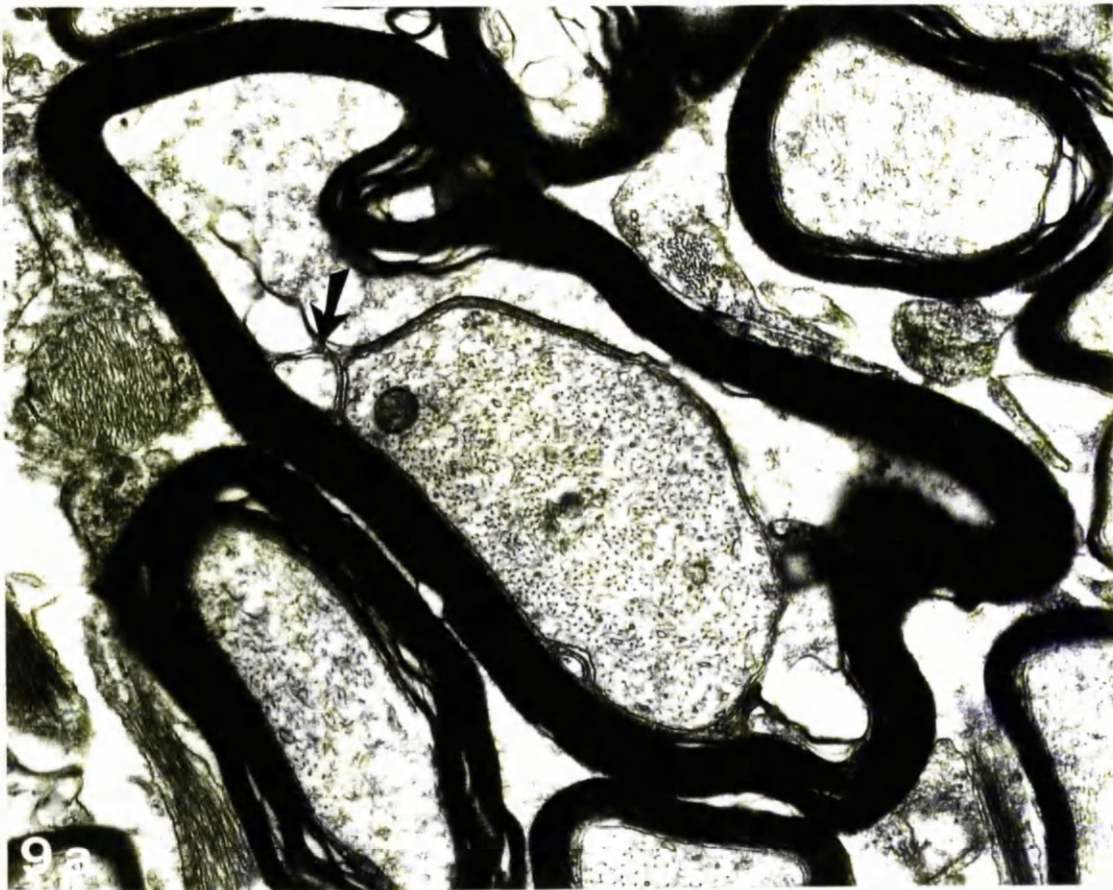


Fig. 10. (a and b) This transverse thin section of a larger nerve fibre ($> 2.0 \mu\text{m}$) after stretch-injury shows (a) a great reduction of axonal calibre and an almost empty periaxonal space (pa) with the axon located centrally. (b) At higher magnification most of the microtubules are grouped at the left side of the axon and there is an overall loss of microtubules. Neurofilaments are highly packed (compacted) and evenly distributed throughout most of the axoplasm. (a) x 40 700, (b) x 101 600.

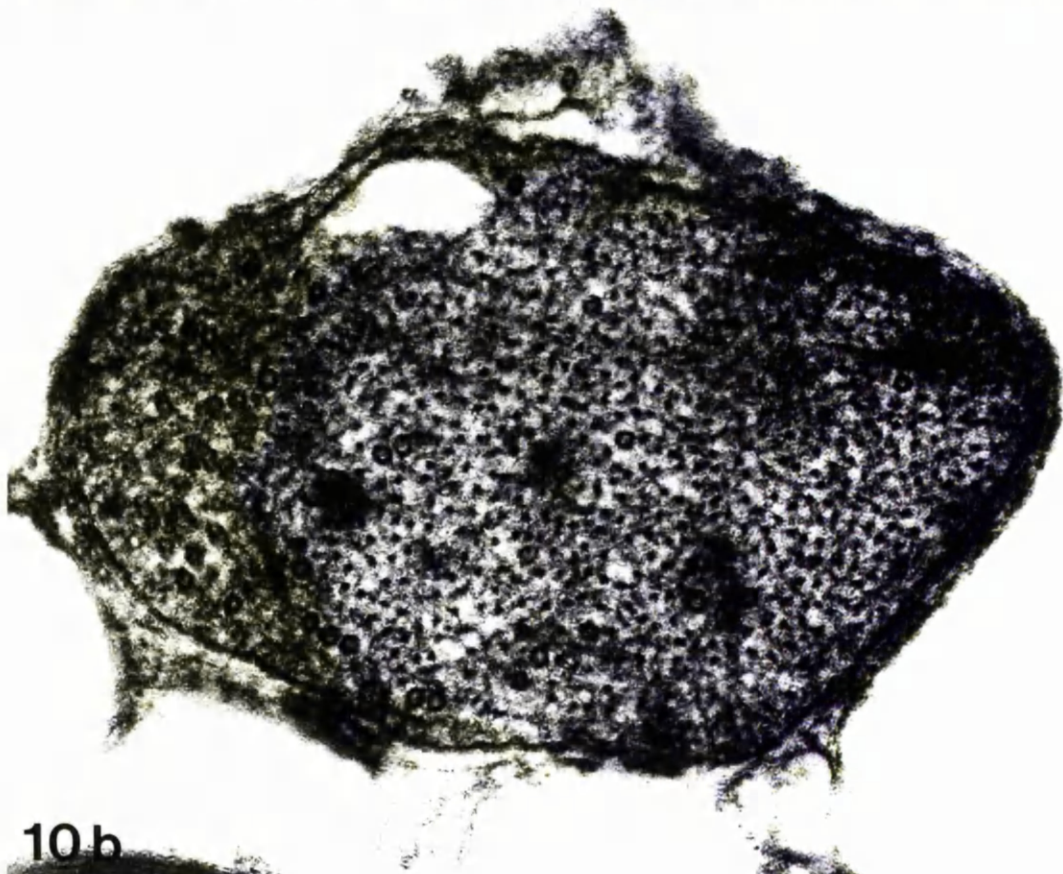
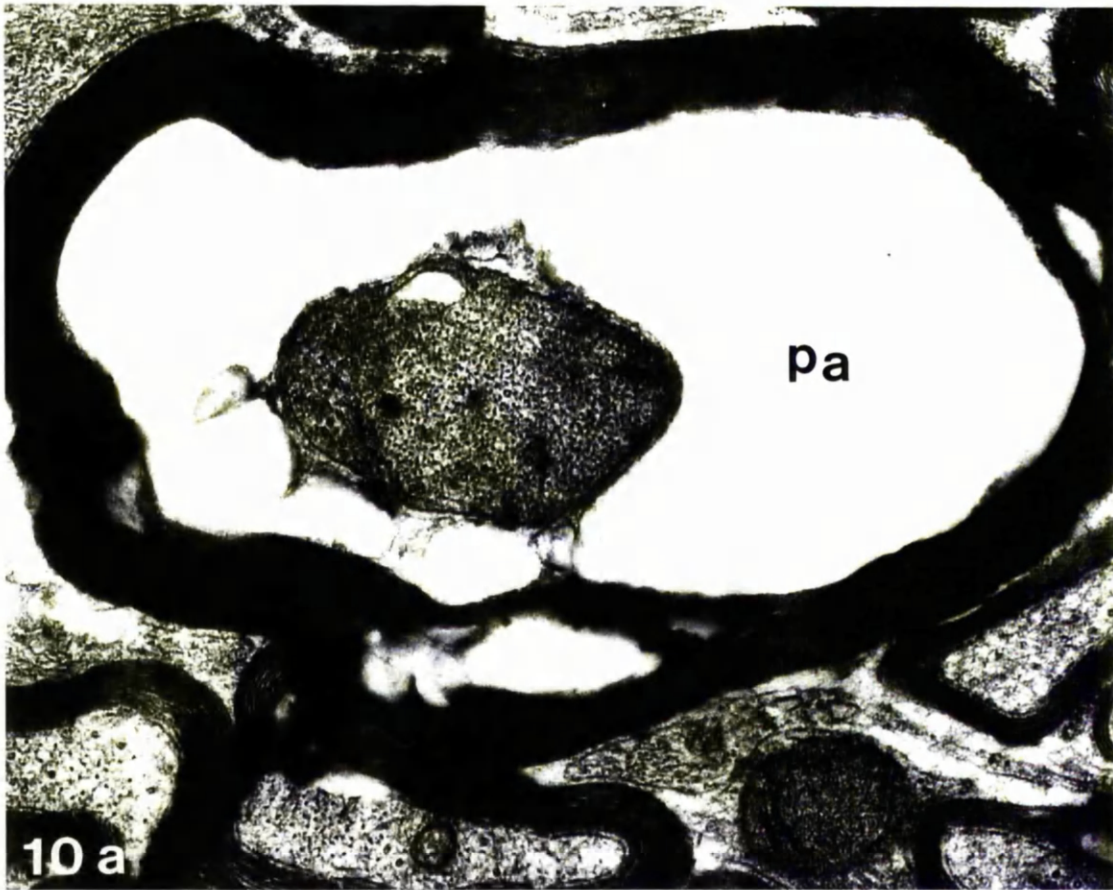
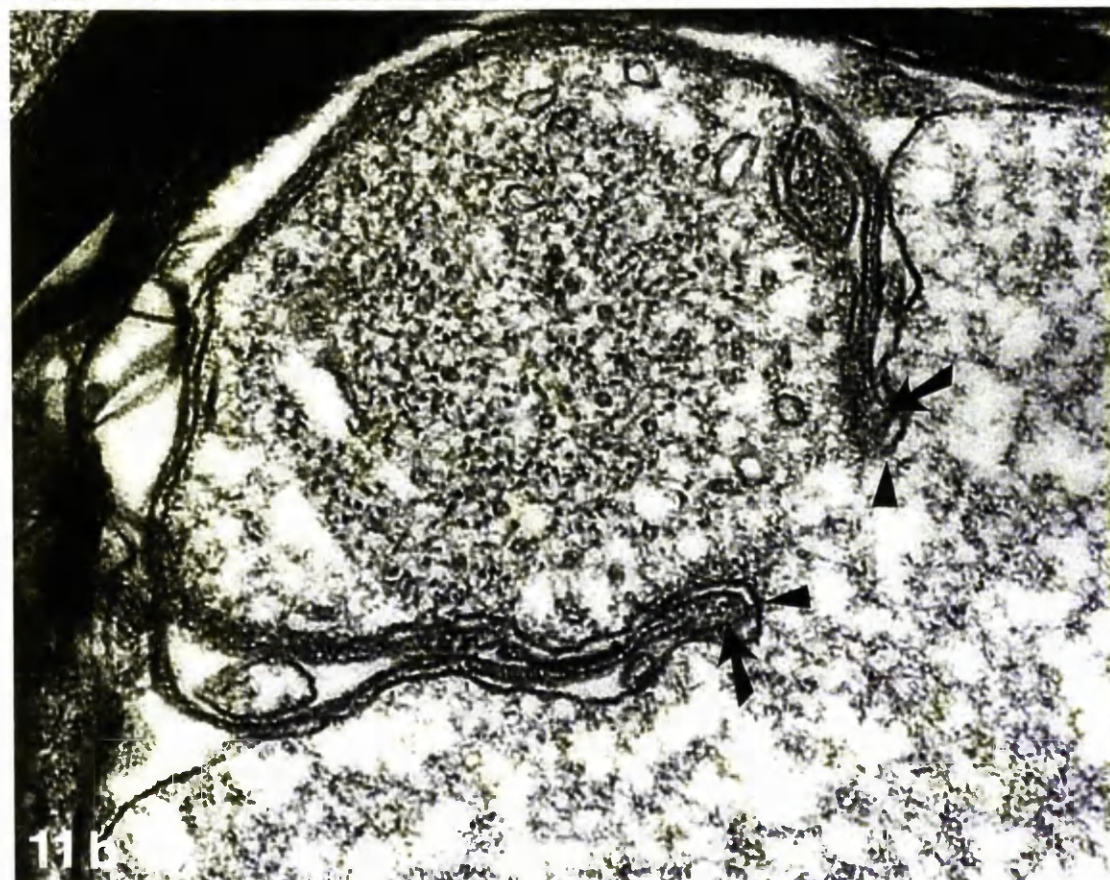
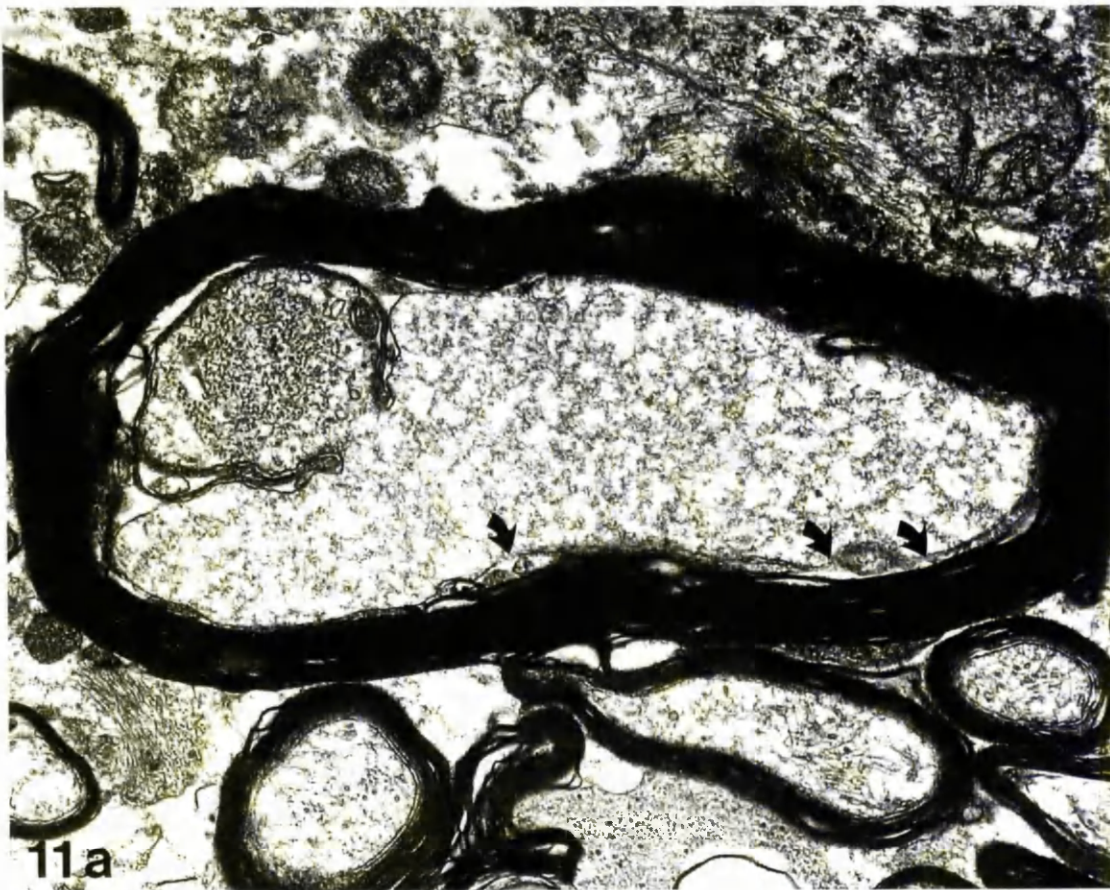


Fig. 11. (a and b) Low and high magnification images of a transverse thin section of a larger nerve fibre ($> 2.0 \mu\text{m}$) after stretch-injury. (a) The top left hand corner of the axon shows a C-shape profile of the axolemma that limits almost all of the axonal cytoskeleton and some vesicles (a and b). The reminder of the axon, the larger portion, contains flocculent material, and the axolemma appears to be broken at some foci (curved arrows). (b) The two ends of the C-shaped axolemma profile (arrowheads) and related glial processes (arrow) approach each other. (a) x 30 150, (b) 93 000.



II.2. Morphometric analysis

II.2.a. Reduction of axonal diameter and the g-ratio in the larger fibres with a periaxonal space

The mean number of the myelin lamellae in fibres with periaxonal space(s) (Maxwell *et al.*, 1995; Jafari *et al.*, 1997) (30.33 ± 1.00 lamellae) (mean \pm SEM) did not change from control values (29.90 ± 0.91 lamellae) obtained in the larger fibres with an axonal diameter greater than $2.00 \mu\text{m}$. According to the relationship between the number of the myelin lamellae and the axonal diameter demonstrated by Hilderbrand and Hahn (1978) - namely, that the number of myelin lamellae increases with the increasing axon size in a curvilinear fashion, these fibres with periaxonal space(s) were categorised as larger fibres with axonal diameter greater than $2.00 \mu\text{m}$ (figs. 9a, b and 10a, b). Thus axons in these fibres were shrunken after stretch-injury. The mean axonal diameter in the fibres with periaxonal space(s) was reduced from $2.61 \pm 0.04 \mu\text{m}$ (mean \pm SEM) in control fibres with axonal diameter greater than $2.00 \mu\text{m}$, to $1.70 \pm 0.06 \mu\text{m}$. This change was statistically highly significant ($t = 11.71$, $df = 3$, $p < 0.002$). But overall, the mean diameter of the myelin sheath limiting fibres ($3.33 \pm 0.15 \mu\text{m}$) did not differ significantly from control values ($3.48 \pm 0.10 \mu\text{m}$) ($t = 0.78$, $df = 3$, n.s.). This resulted in loss of close relationship of the axolemma with the myelin sheath (figs. 9a, b and 10a, b).

The g-ratio in the larger control fibres (axonal diameter $> 2.00 \mu\text{m}$) was found to be 0.74 ± 0.006 . The g-ratio in fibres with periaxonal space(s) was reduced from 0.74 ± 0.006 to 0.50 ± 0.007 , that is a 32.4% reduction. The statistical analysis showed that the change in g-ratio was highly significant ($t = 26.84$, $df = 3$, $p < 0.001$).

II.2.b. G-ratio and the number of myelin lamellae in the larger fibres with intramyelinic spaces

The g-ratio (0.73 ± 0.006) in the larger fibres (axonal diameter $> 2.00 \mu\text{m}$) with intramyelinic spaces did not change from control values (0.74 ± 0.006). In these fibres the number of myelin lamellae (29.70 ± 0.62) was within the range of control values (29.90 ± 0.91) after stretch-injury.

II.2.c. Changes in the spacing of neurofilament and microtubule and their density per unit area of the axoplasm

As described above larger fibres fell into two subclasses. Either those fibres with intramyelinic spaces (fig. 8a, b), or those in which periaxonal space(s) occurred (Figs. 9a, b and 10a, b). The changes in cytoskeletal relationships in the axons of these two subclasses of fibre differed both from those in the smallest axons and between the two groups.

II.2.ci. Larger fibres with intramyelinic spaces

In the axons of those nerve fibres within which intramyelinic spaces occurred neurofilament spacing was increased significantly ($p < 0.05$) by 34.7% (table 7; page 120). This increased spacing was reflected in the neurofilament density per unit area of axoplasm which was decreased by 58.9% (table 8; page 121). This reduction in density per unit area was highly significant ($t = 15.21$, $df = 3$, $p < 0.001$).

There was also a 57.2% increase in microtubule spacing from control values ($p < 0.002$), and a 56.5% reduction in the density of microtubules per unit area. Statistical

analysis demonstrated that the reduction in microtubule density per unit area of axoplasm was highly significant ($t = 10.82$, $df = 3$, $p < 0.002$).

II.2.cii. Larger fibres with periaxonal space(s)

In the larger fibres in which periaxonal spaces occurred there was a 22.9% statistically significant reduction in spacing between neurofilaments (table 7; page 120) ($t = 9.23$, $df = 5$, $p < 0.001$). There was a 86.2% significant increase in their density per unit area of axoplasm (table 8; page 121) ($t = 8.21$, $df = 2$, $p < 0.02$). These results, therefore, provided evidence for compaction of neurofilaments (figs. 9a, b and 10a, b) in the larger fibres in which periaxonal space(s) occurred after stretch-injury.

Changes in respect of microtubules were the opposite with a 25.9% significant increase in spacing (table 7; page 120) ($t = 4.61$, $df = 2$, $p < 0.05$) or a 52.8% significant decrease in density per unit area (table 8; page 121) ($t = 11.55$, $df = 2$, $p < 0.01$).

Thus, after stretch-injury, in the larger nerve fibres with intramyelinic spaces, there was an increased spacing between neurofilaments and between microtubules. However, there was a reduction in spacing between neurofilaments, but an increase in spacing between microtubules in nerve fibres in which a periaxonal space(s) occurred. Thus increased microtubule spacing occurred even in fibres where neurofilament compaction occurred 4 hours after stretch-injury. There was, therefore a different response in axons of nerve fibres in which intramyelinic and periaxonal spaces occurred after stretch-injury with regard to the spacing and density per unit area of axoplasm for neurofilaments. But for microtubules there was a parallel change in that

microtubules became more widely spaced in both situations where either intramyelinic spaces or periaxonal space(s) occurred after stretch-injury.

II.2.d. Changes in the total number of neurofilaments and microtubules in axons

Counts of the means of the total number of neurofilaments and microtubules in cross-sections of 150 control and 30 stretch-injured larger axons from 3 control and 3 experimental animals, including 15 axons in fibres with a periaxonal space(s) provided the data given in table 9 (page 121).

II.2.di. Larger axons with intramyelinic spaces

In larger axons in which intramyelinic spaces occurred after stretch-injury and which demonstrated an increase in neurofilament spacing (table 7; page 120), there was a 27% loss in the number of neurofilaments from the control value. This change (table 9; page 121) was statistically significant ($t = 6.67$, $df = 2$, $p < 0.05$). Thus the decrease in the density (table 8; page 121) of neurofilament per unit area was correlated with a loss of their number.

There was a 55.4% reduction in the number of microtubules. This change was statistically significant ($t = 7.08$, $df = 2$, $p < 0.02$). This means that the increase in the spacing and decrease in the density per unit area of microtubules was due to a reduction in their number within stretch-injured axons with intramyelinic spaces (tables 7, 8 and 9; pages 120 and 121).

II.2.dii. Larger axons with periaxonal space(s)

Although there was a 14.4% increase in the number of neurofilaments in the larger fibres with periaxonal space(s) in which neurofilament compaction occurred within the axon, this change was not statistically significant ($t = 2.91$, $df = 3$, n.s.). Therefore, the decrease in spacing and increase in neurofilament density per unit area of axoplasm was principally due to compaction of neurofilaments, but not a change in their number (tables 7, 8 and 9; pages 120 and 121).

However, in these fibres there was a highly significant loss (by 72.2%) in the number of axonal microtubules ($t = 9.78$, $df = 2$, $p < 0.02$). Thus the increase in the spacing and decrease in the density per unit area of axoplasm principally resulted from a loss in their number (tables 7, 8 and 9; pages 120 and 121).

III. Analysis of Longitudinal Sections

A differential axonal cytoskeletal response was also suggested by observation of longitudinal thin sections in the smallest and the larger nerve fibres 4 hours after stretch-injury to guinea pig optic nerve. Compaction of neurofilaments occurred in the smallest nerve fibres that formed central core of neurofilaments over a length of several microns (fig. 12). In larger fibres a variety of cytoskeletal changes occurred both in different fibres and over part of the length of the same fibre. From the examination of these changes in different nerve fibres or along the length of a single nerve fibre it appeared that, 1. At a minimal level of injury the axonal calibre is uniform, but there is misalignment of the cytoskeleton, especially microtubules which had a helical rather than linear disposition within the axoplasm (fig. 13), 2. At a greater level of injury there is variation in axonal calibre over the length of the nerve fibre (fig.

14a). This may be comparable to the ‘beading’ noted in nerve fibres after ‘light stretch’ (Ochs and Jersild 1987, Ochs *et al.*, 1994, 1996). In the nerve fibre in figure 14a axonal diameter varies from a minimum of 0.8 μm to a maximum of 2.03 μm . In this fibre where the axonal diameter is at minimum (fig. 14a) the axonal cytoskeleton contains a large number of closely packed neurofilaments aligned parallel to the longitudinal axis of the axon (fig. 14c). The myelin sheath is greatly disrupted and myelin lamellae are separated (fig. 14a). There was a low number of microtubules in this portion of the axon (fig. 14c). On the other hand, in the region where the axonal diameter is maximal (the area of dotted lines in figure 14a) the longitudinal alignment of the axonal cytoskeleton is lost where some neurofilaments run obliquely through the axoplasm (arrow in fig. 14 a,b). At this region the spacing between neurofilaments is greatly increased (fig. 14a, b), 3. At the greatest level of damage there were multiple foci of compaction of neurofilaments linearly arranged along the length of the axon (fig. 15). Foci of compaction of neurofilaments, about 1.50 to 2.50 μm in length were scattered along the length of the axon (fig. 15). These foci were separated by regions about 4.5 μm long where neurofilament spacing appear to be normal (fig. 15). The nerve fibre in figure 16 shows that where neurofilament compaction occurred the axolemma has lost its continuity large holes were present in both longitudinal (fig. 16) and transverse sections (fig. 17). The loss of the close relationship of the axolemma with the axonal cytoskeleton and myelin sheath is observed in both longitudinal (fig. 16) and transverse section (fig. 17). In longitudinal section (fig. 16) fragments of the axolemma extended into the enlarged periaxonal space. Further at foci of compaction of neurofilaments and fragmentation of axolemma there was gross distortion of the myelin sheath (fig. 16) to form a “balloon-like” expansion (Jafari *et al.*, 1997) (figs. 15

and 16). In transverse section, however, it is clear that recognisable neurofilaments are retained at these sites of compaction (fig. 17).

Fig. 12. A longitudinal thin section of a small nerve fibre ($< 0.5 \mu\text{m}$) after stretch-injury. The axonal cytoskeleton consists of neurofilaments forming a central core within the axoplasm. x 121 750.

Fig. 13. A longitudinal thin section of a damaged larger nerve fibre in which the components of the axonal cytoskeleton are no longer longitudinally oriented. Microtubules (arrow) have a helical disposition within the central region of the axoplasm. The peripheral part of the axoplasm is electron lucent. x 63 450.

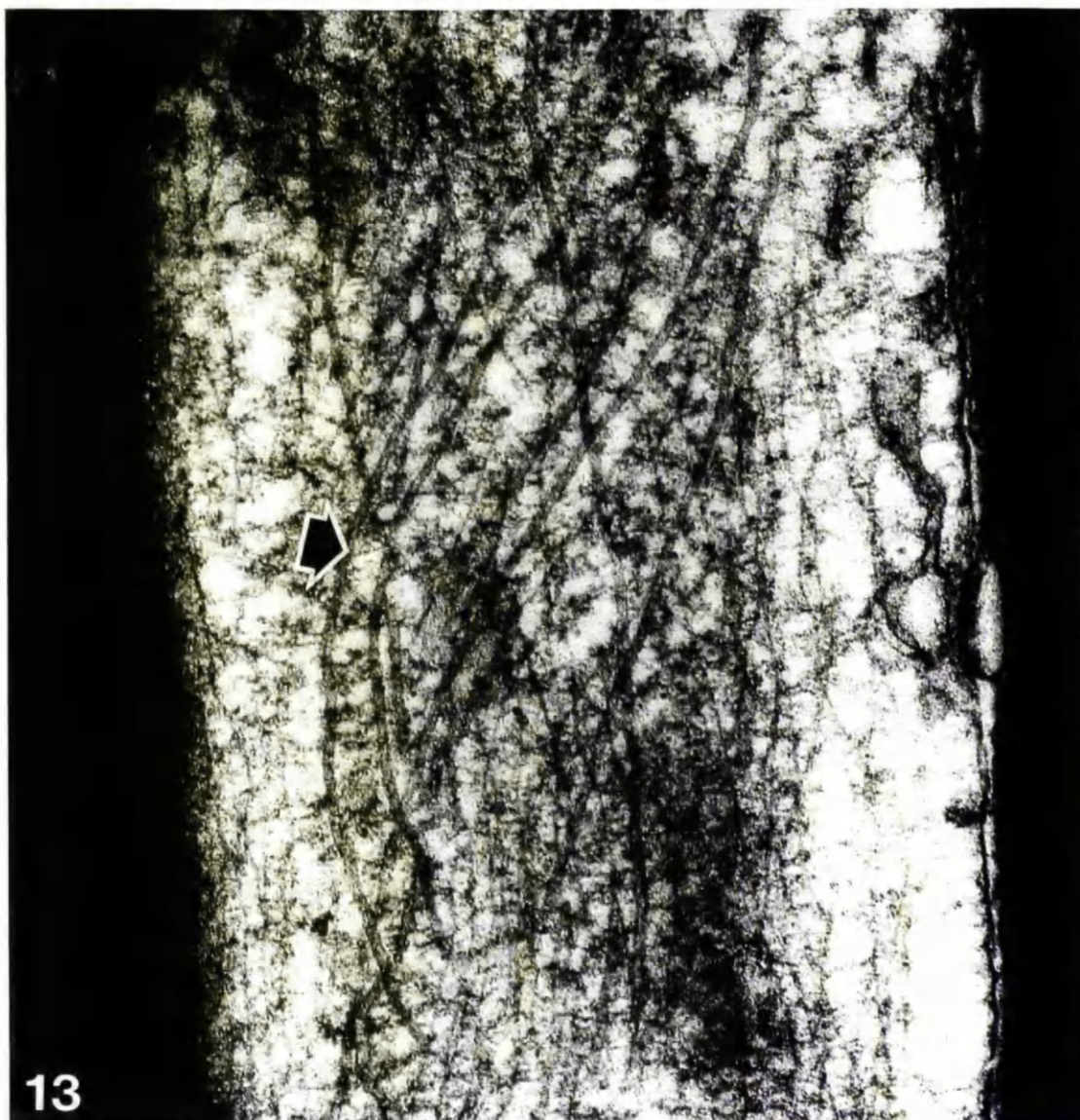
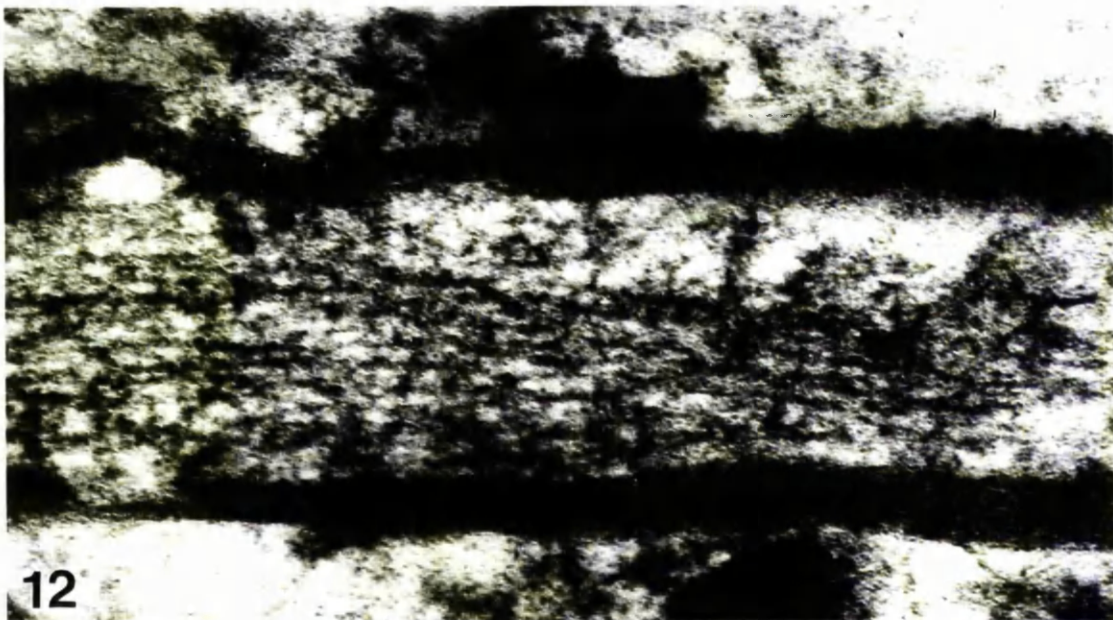
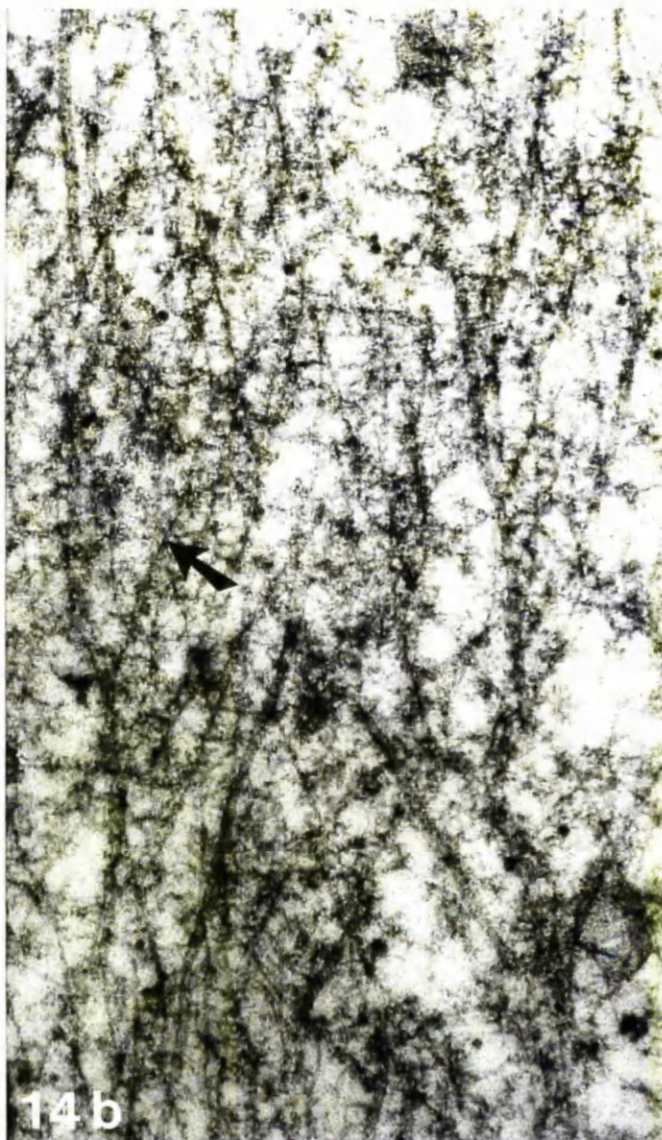


Fig. 14. (a) A photomontage of a longitudinal section of the internode of a damaged larger nerve fibre. There is a variation in axonal calibre. The greatest disruption of the myelin sheath occurs at the site of minimum axonal diameter. x 21 900. Two parts of this fibre are shown at higher magnification. (b) The region of maximum diameter (area between the dotted lines). Here there is loss of a linearly organised cytoskeleton, rather neurofilaments have no preferred orientation. Indeed some neurofilaments run obliquely (arrow) through the axoplasm. (c) The region of minimum diameter. Here neurofilaments are basically longitudinally oriented but with a decreased spacing between them. (b) x 73 200, (c) x 72 250.

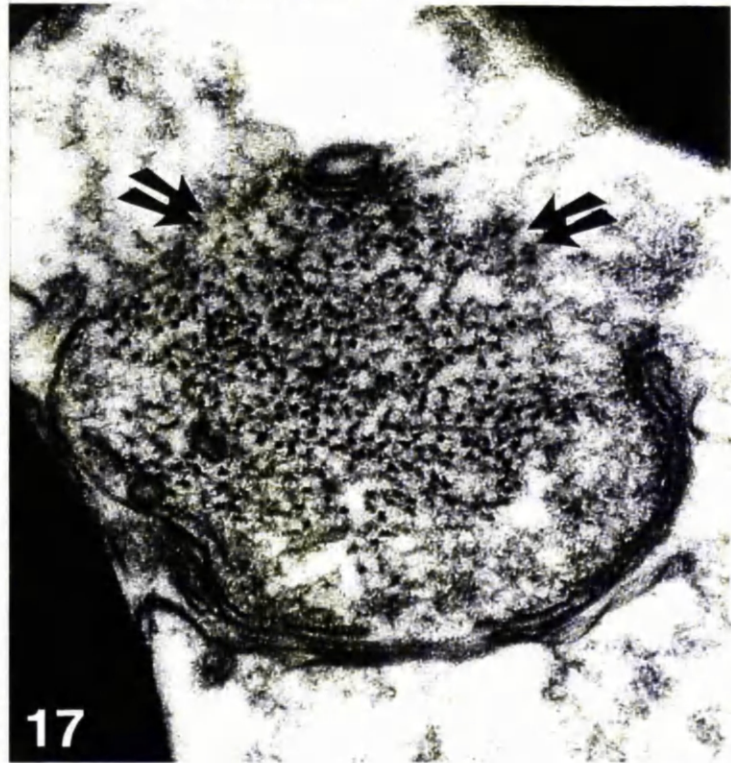


Figures 15, 16 and 17 demonstrate longitudinal and transverse sections through severely damaged nerve fibres.

Fig. 15. A photomontage of a longitudinal section of a nerve fibre showing a number of foci of compaction of neurofilaments (arrows), associated with an increase in fibre diameter to form a balloon-like expansion, separated by regions where the axon/fibre has a more normal structure. x 18 700.

Fig. 16. A longitudinal section of a nerve fibre at a balloon-like expansion showing compaction of neurofilaments. At this region there is disruption of the axolemma such that fragments extend into the periaxonal space (curved arrow) and the neurofilament core is no longer limited by the axolemma (double arrows). There is also disruption of the myelin sheath to form irregular figures limiting the periaxonal space. x 19 350.

Fig. 17. This transverse section of a nerve fibre also shows disruption of the axolemma (double arrows) at a focus of neurofilament compaction. Both longitudinal (fig. 16) and transverse sections (this figure) show that at foci of compaction of neurofilaments, only the neurofilamentous components of the axonal cytoskeleton are present. x 83 600.



CHAPTER FIVE

ANALYSIS OF CHANGES IN THE INTERMEDIATE BIN SIZE FIBRES WITH AN AXONAL DIAMETER RANGING FROM 0.51 TO 2.00 μM

I. Morphological analysis of transverse sections

I.1. Control fibres

In the control fibres of axons within the bin sizes of 0.51-1.00, 1.01- 1.5 and 1.51-2.00 μm , the myelin sheath lamellae were smooth in shape and arranged regularly (figs. 18, 20, and 22a, b). The profile of axolemma was smooth and regular and clearly distinguished. The axolemma had a close association with the myelin sheath. There was a relatively low number of neurofilaments and microtubules in the smaller axons, and a higher number in the larger axons (figs. 18, 20 and 22a, b).

I.2. Stretch-injured fibres

I.2.a. Changes in the axolemma and myelin sheath in all three bin sizes

Examination of experimental fibres 4 hours after stretch-injury to the optic nerve showed that the changes in the myelin sheath and the axolemma were similar in different fibres within different axonal bin sizes. The differences in changes were between the cytoskeletal components of the axoplasm in different axonal bin sizes. The myelin lamellae in some parts of the myelin sheath were separated from each other to form intramyelinic spaces (Figs. 19, 21 and 23a). In some fibres the myelin lamellae had a wavy appearance in some regions of the myelin sheath (fig. 19). At some sites the axolemma became irregular in shape and lost its close association with the myelin sheath (figs. 19, 21 and 23a).

I.2.b. Changes in the axonal cytoskeleton

I.2.bi. Fibres with an axonal diameter within the bin size of 0.51-1.00 μm

Examination of the axonal cytoskeleton revealed that the number of neurofilaments appeared to be higher than control. These neurofilaments were usually localised and compacted together in a part of the axoplasm (fig. 19). Microtubules were usually located at the periphery of the axon.

I.2.bii. Fibres with an axonal diameter within the bin size of 1.01-1.50 μm

Compared to the axons in the previous bin size (fig. 19), neurofilaments in the axon of these fibres were still localised and grouped together, but the apparent degree of the compaction was less (fig. 21). The microtubules were now scattered throughout the axoplasm, but with a slightly greater concentration at the periphery. The number of microtubules appear to be less than in control axons (fig. 20) within the same range size.

I.2.biii. Fibres with an axonal diameter within bin size of 1.51-2.00 μm

In the axons of these fibres cytoskeletal components were scattered throughout the axoplasm. The neurofilaments were not compacted (fig. 23a, b). The spacing between neurofilaments and between microtubules in the axoplasm appeared to be wider. Comparison with control axons (fig. 22a, b) suggested some loss in the number of both neurofilaments and microtubules (fig. 23a, b).

Fig. 18. A transverse thin section of a control myelinated axon with a diameter between 0.51 and 1.00 μm . The myelin sheath lamellae are smooth in shape and regularly arranged. Neurofilaments (arrow) and microtubules (arrowhead) occur throughout the axoplasm. x 87 300.

Fig. 19. A transverse thin section of a myelinated axon between 0.51 and 1.00 μm in diameter after stretch-injury. The myelin lamellae are dissociated or loosened and some spaces are formed between them. The axolemma has an irregular shape and has lost its close association with the myelin sheath at some sites (curved arrows). Neurofilaments are compacted and grouped close to the centre of the axoplasm, while microtubules tend to occur at the periphery. The number of neurofilaments is higher than in comparable control axons (fig. 18). x 99 300.

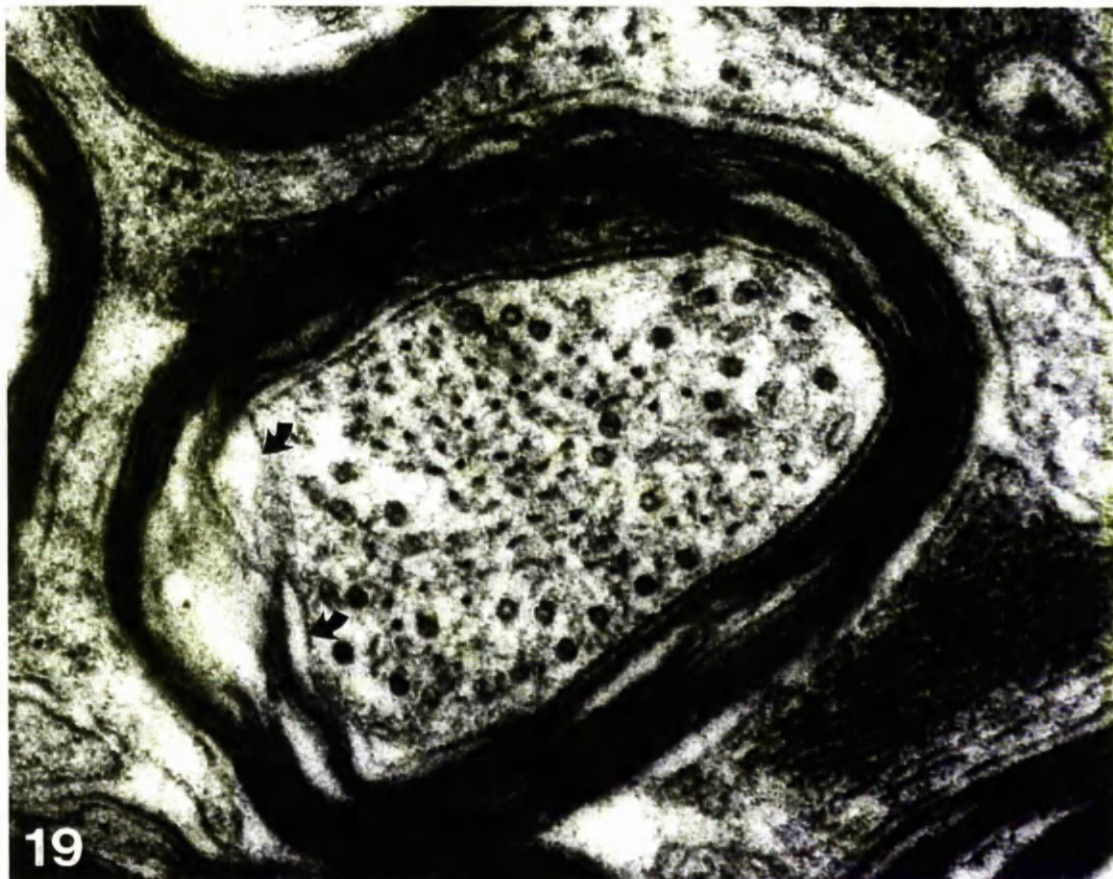
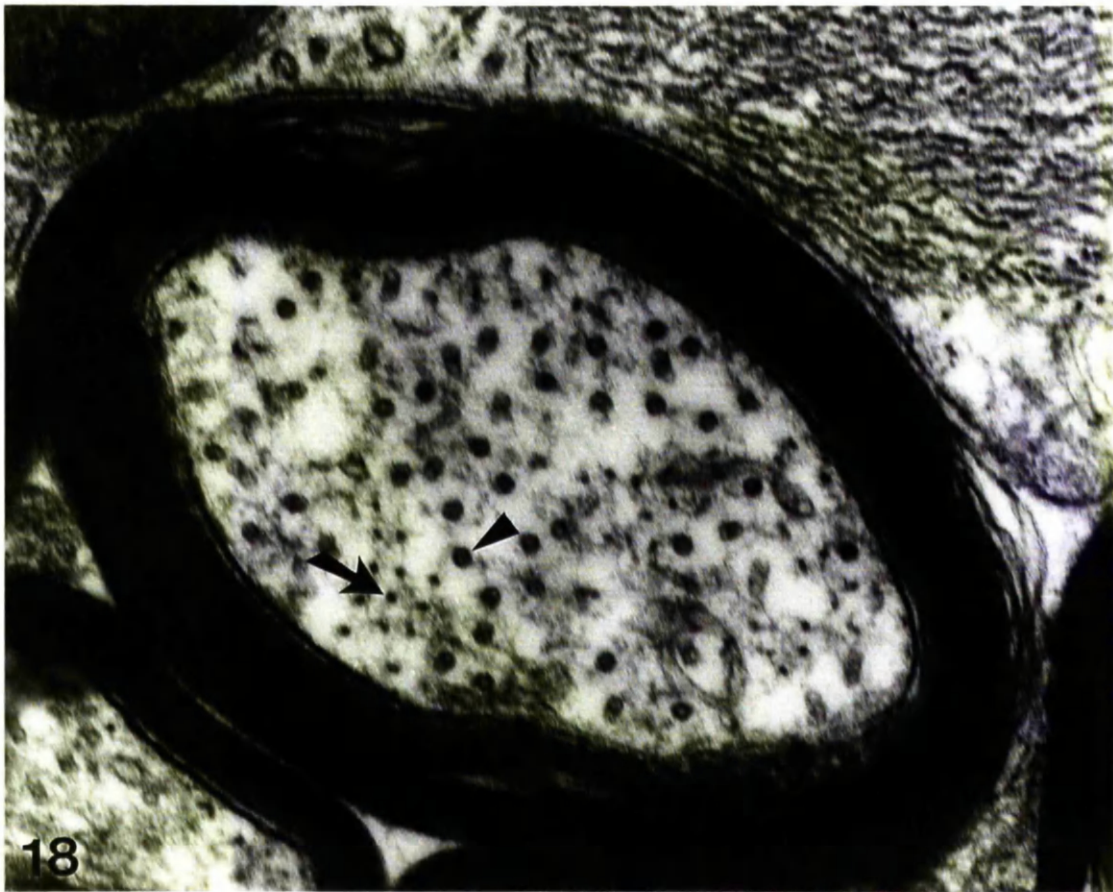


Fig. 20. A transverse thin section of a control myelinated axon with a diameter between 1.01 and 1.50 μm . There are greater number of both neurofilaments and microtubules than in smaller axons (compare with fig. 18). x 69 600.

Fig. 21. A transverse thin section of a myelinated axon with a diameter between 1.01 and 1.50 μm after stretch-injury. The axonal profile is irregular and there is separation of the axolemma from the myelin sheath at some sites (curved arrows). Neurofilaments, with a reduced spacing between them, form a central group. The number of microtubules is lower than in comparable control axons (fig. 20) and are scattered throughout the axoplasm with a slightly greater concentration at the periphery. x 96 750.

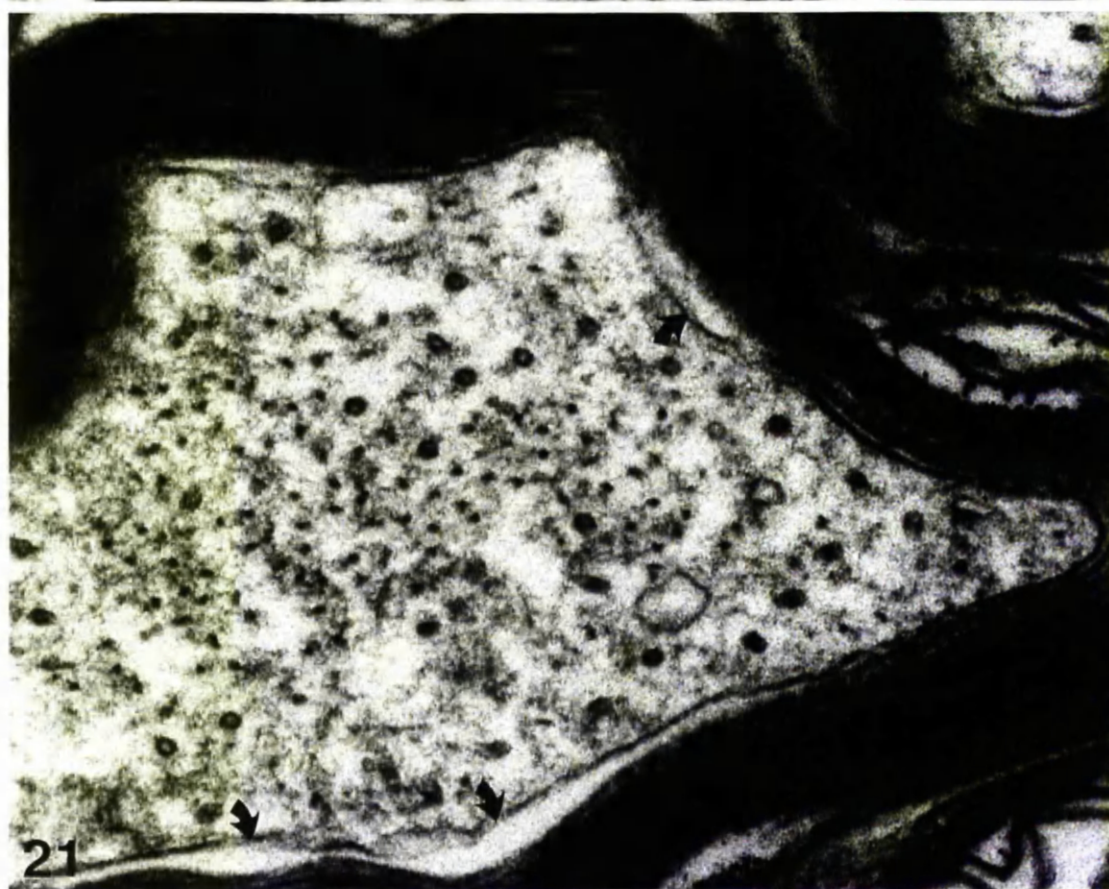
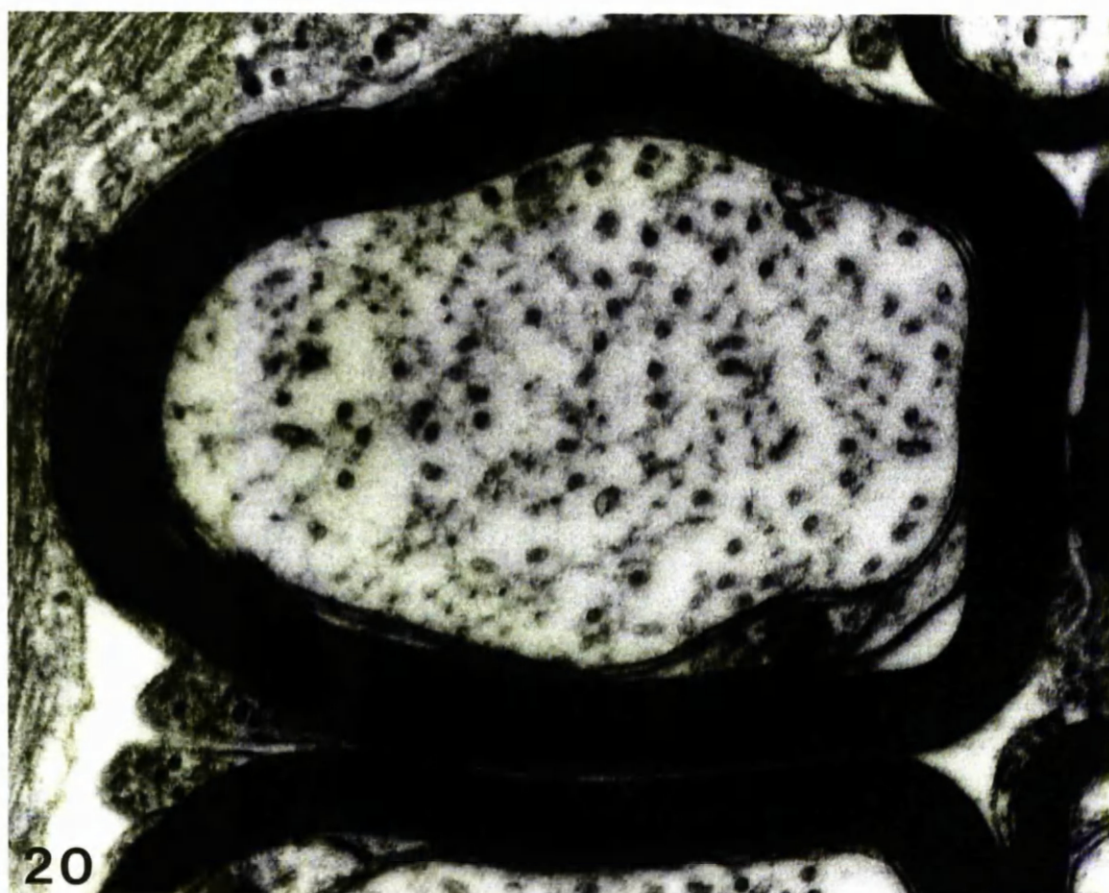


Fig. 22. (a and b) Low and high magnification images of a transverse thin section of a central myelinated, control axon with a diameter between 1.51 and 2.00 μm . Neurofilaments outnumber microtubules. There is a greater number of neurofilaments than in smaller axons (figs. 18 and 20) and these are distributed throughout the axoplasm. Microtubules are numerous. (a) x 47 250, (b) x 110 500.

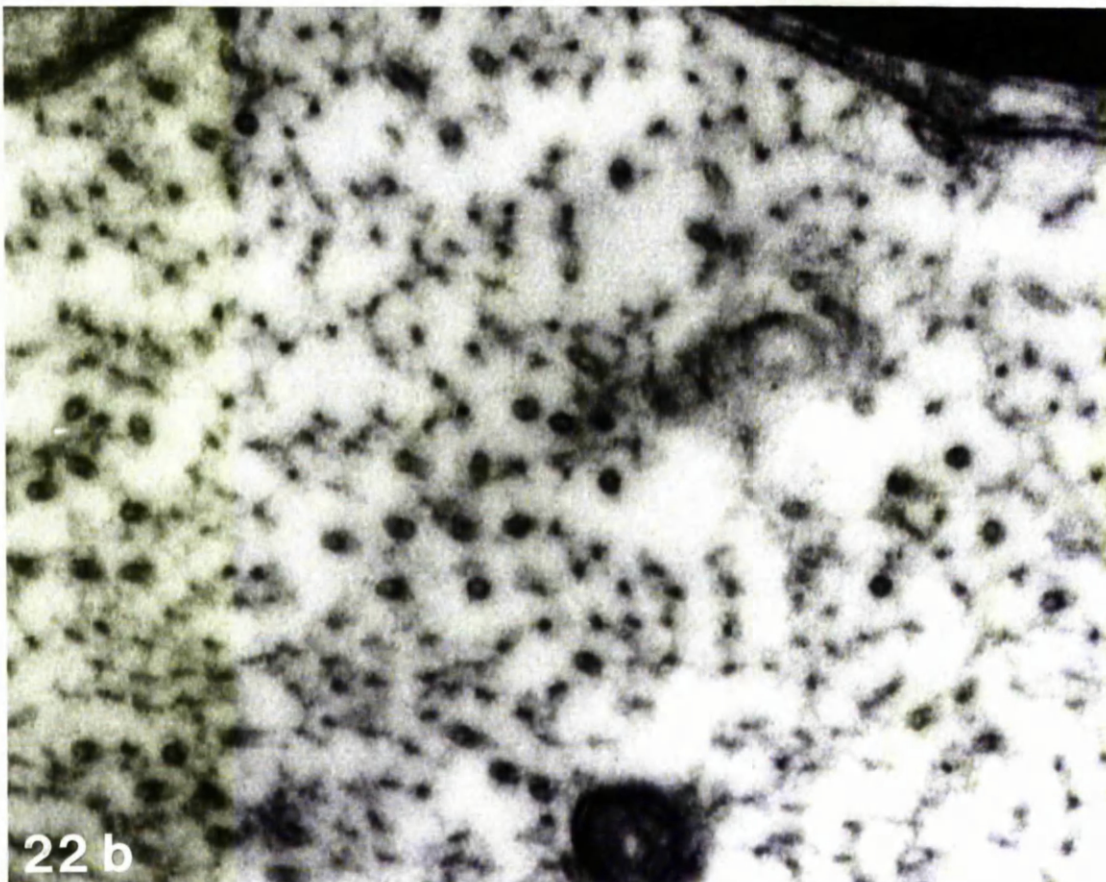
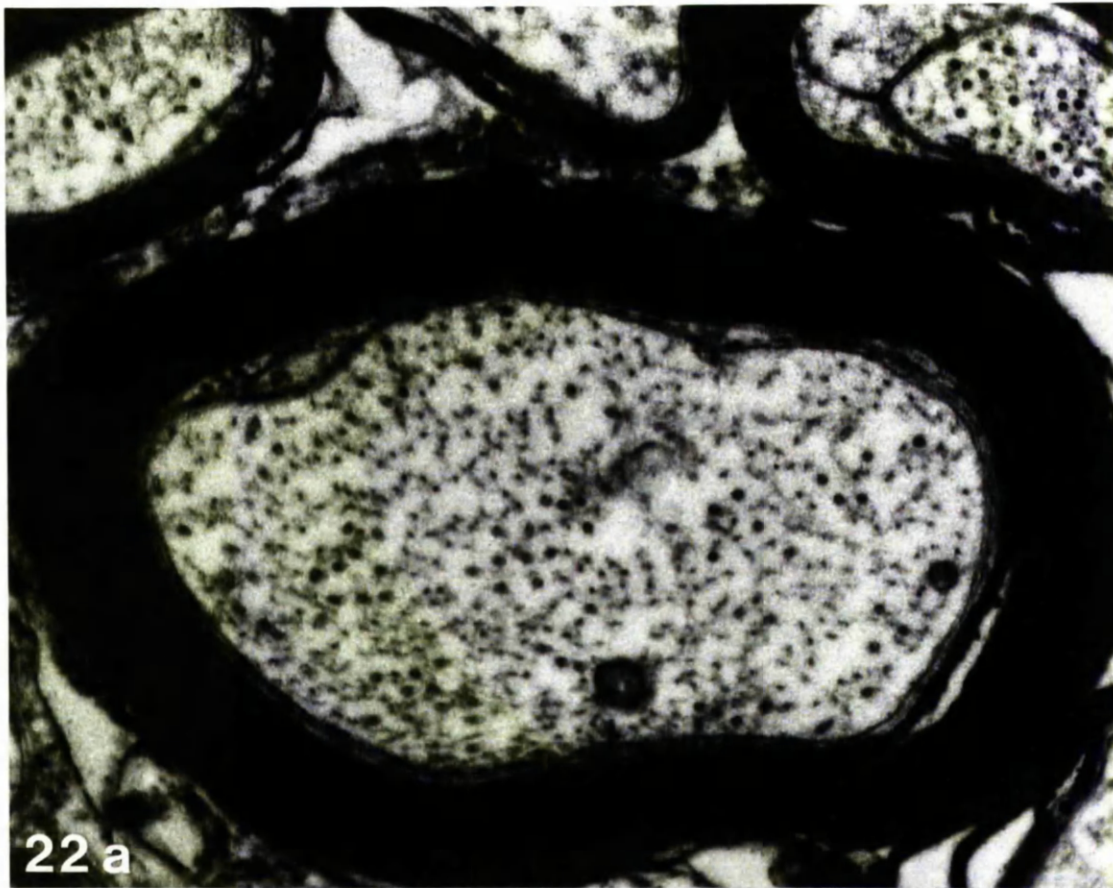
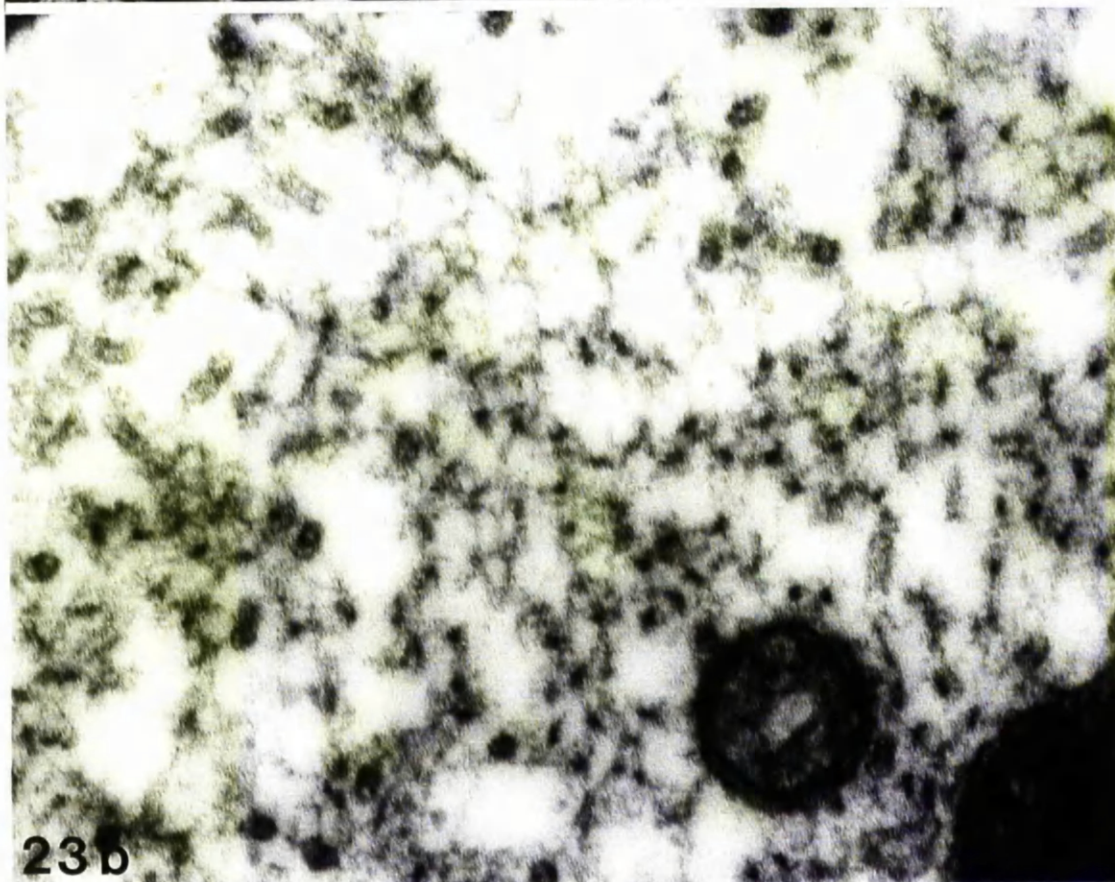
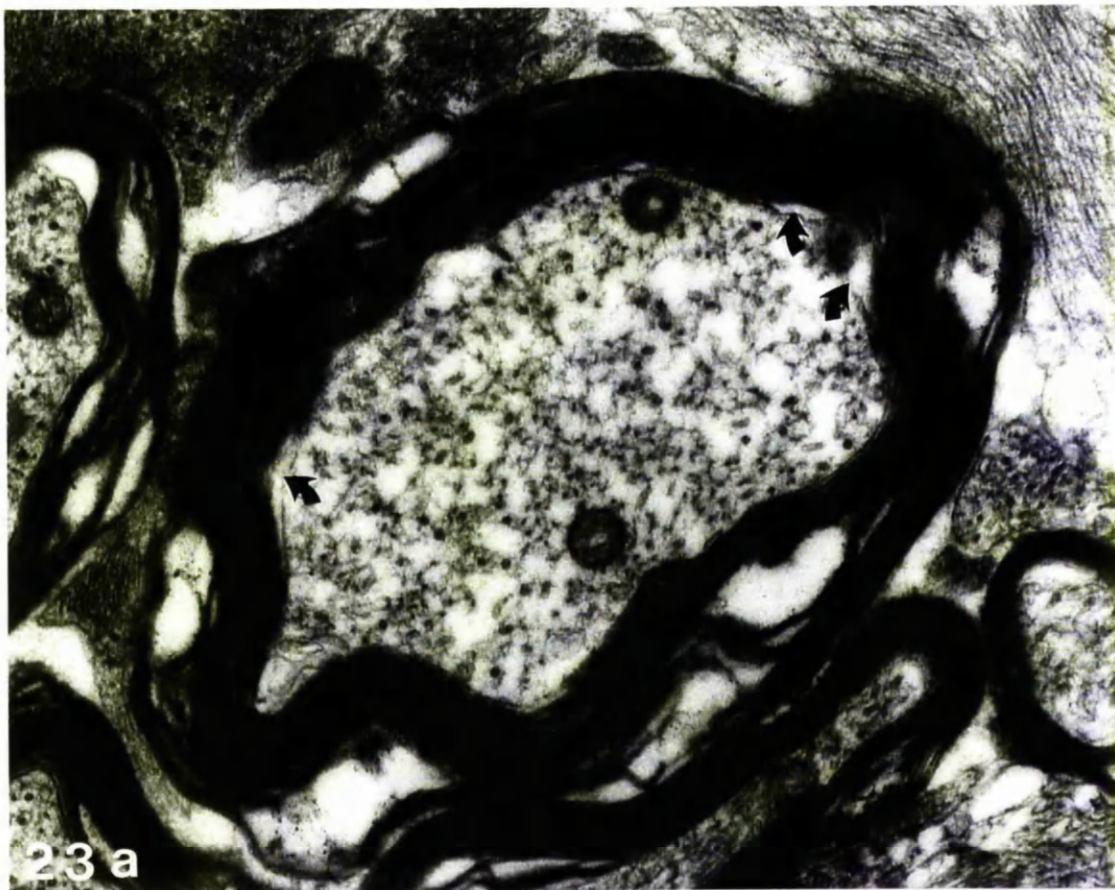


Fig. 23. (a and b) Low and high magnification images of a transverse thin section of a myelinated axon with a diameter between 1.51 and 2.00 μm after stretch-injury. The axonal and myelin sheath profiles are irregular, and there are some spaces between myelin lamellae. There are some sites of separation of the axolemma from the myelin sheath (curved arrows). Neurofilaments are not distributed uniformly throughout the axoplasm. The number of both neurofilaments and microtubules is lower than in a comparable control axon (fig. 22 a and b). (a) x 38 550, (b) x 130 000.



II. Morphometric analysis of transverse sections

II.a. Changes in the number or proportion of the axons and fibre parameters

As described earlier there was a decrease in the number of the axons falling within bin size of 0.51-1.00 μm by 10.5% (table 1; pages 103 and 104). Application of χ^2 test showed that this decrease was statistically significant ($p < 0.05$). There was not any significant change in the number of the axons in the bin sizes of 1.01-1.50 μm and 1.51-2.00 μm (table 1; page 104) ($p > 0.05$).

There was not any significant change in the number of the nerve fibres in the middle segment of the injured optic nerve (table 2; pages 105 and 106).

The g-ratio and fibre parameters including:- fibre diameter, axon diameter, sheath thickness and number of myelin lamellae did not alter after the injury, and the obtained values fell within the control range (table 10). In order to measure the exact myelin sheath thickness and axon diameter, the normal periaxonal space (see page 5) was not measured. This is the reason that there is a small difference between overall fibre diameter and the fibre diameter obtained from adding the myelin sheath thickness to the axon diameter (table 10).

Table 10

Comparison of fibre parameters in myelinated nerve fibres with axons falling within 0.50 μm wide bins of internodal axonal diameter ranging from 0.51 to 2.00 μm (Mean \pm SEM).

	Control			Experimental		
Bin size (μm)	0.51-1.00	1.01-1.50	1.51-2.00	0.51-1.00	1.01-1.50	1.51-2.00
Axon diameter	0.81 \pm 0.01	1.2 \pm 0.02	1.72 \pm 0.03	0.78 \pm 0.02	1.2 \pm 0.02	1.72 \pm 0.07
Fibre diameter	1.15 \pm 0.03	1.73 \pm 0.01	2.46 \pm 0.05	1.07 \pm 0.3	1.67 \pm 0.08	2.41 \pm 0.14
Sheath thickness	0.29 \pm 0.01	0.46 \pm 0.02	0.66 \pm 0.03	0.27 \pm 0.04	0.43 \pm 0.07	0.63 \pm 0.08
Number of lamellae	9.53 \pm 0.22	15.57 \pm 0.22	22.33 \pm 0.97	9.13 \pm 0.62	14.27 \pm 0.64	21.33 \pm 0.63
G-ratio	0.70 \pm 0.007	0.69 \pm 0.009	0.70 \pm 0.003	0.71 \pm 0.03	0.72 \pm 0.03	0.72 \pm 0.02

II.b. Relation between myelin lamellae and axon diameter

The data obtained from 10 axons in each axonal bin size (ranging from 0.00-3.00) from each control optic nerve, was used to plot a graph of axonal diameter against the number of myelin lamellae (fig. 27). The graph demonstrated that the number of myelin lamellae was related to the axon diameter according to a curvilinear function. The correlation coefficient of the slope was $r^2 = 0.86$. The number of myelin lamellae increased regularly with the increase in axon diameter up to an axon diameter of about 2.30 μm , which correspond to 29 myelin lamellae in the myelin sheath. Above this size the relation between number of myelin lamellae and axon diameter was less strict and the number of myelin lamellae increased progressively less with increasing axonal diameter. This graph was used to predict the axonal diameter, according to the number of myelin lamellae, in the stretch-injured fibres when they were sampled. The relation

between the number of the myelin lamellae and axon diameter obtained from injured axons in the middle range size revealed no deviation from control values (table 10).

II.c. Changes in the spacing between neurofilaments and between microtubules

II.ci. Interneurofilament spacing

Interneurofilament spacing in the axons in the bin sizes of 0.51-1.00, 1.01-1.50 and 1.51-2.00 μm was decreased by 41.6%, 35.2% and 17.5% respectively (table 11). The decrease in the first and second bin size were statistically significant ($t = 5.16$ $df = 2$, $p < 0.05$) ($t = 5.01$, $df = 2$, $p < 0.05$), but the change in the third group was not significant ($t = 1.40$, $df = 2$, n.s.). As the results show (table 11), neurofilaments were compacted after stretch-injury, and this compaction had an inverse relationship with the axon size meaning that, neurofilament compaction decreased concomitant with the increase in axon size from smaller axonal bin size to larger ones. In the axons with the bin size of 0.51-1.00 μm and 1.01-1.50 μm there was a compaction of neurofilaments, but spacing between neurofilaments in the axons within a bin size of 1.51-2.00 μm was close to normal.

Table 11

Mean spacing (\pm SEM) between neurofilaments and between microtubules (nm) in axons falling within 0.50 μm wide bins of internodal axonal diameter ranging from 0.51 to 2.00 μm .

	Control			Experimental		
Bin size (μm)	0.51-1.00	1.01-1.50	1.51-2.00	0.51-1.00	1.01-1.50	1.51-2.00
Neurofilaments	116.1 \pm 8.6	111.3 \pm 7.8	96.6 \pm 12	67.84 \pm 3.7	72.14 \pm 0.6	79.67 \pm 2.8
Change from control (%)				- 41.57	- 35.18	- 17.52
Microtubules	134.97 \pm 2.1	151.7 \pm 9.6	147.5 \pm 7.4	141.3 \pm 9.2	179.6 \pm 14	204.3 \pm 12
Change from control (%)				+ 4.7	+ 18.4	+ 38.51

II.cii. Intermicrotubule spacing

Contrary to the decrease in interneurofilament spacing, intermicrotubule spacing was increased by 4.7%, 18.4% and 38.5% in axons of bin sizes of 0.51-1.00, 1.01-1.50 and 1.51-2.00 μm respectively (table 11). The increased spacing in the first and second axonal bin sizes was not significant ($t = 0.67$, $df = 2$, n.s.) ($t = 1.65$, $df = 3$, n.s.). But the increase was statistically significant in the third axonal bin size ($t = 4.12$, $df = 3$, $p < 0.05$). The results demonstrated that, with an increase in axonal size from bin size of 0.51 to 2.00 μm there was an associated increase in the spacing between microtubules after stretch-injury to axons.

II.d. Changes in neurofilament and microtubule density per unit area

II.di. Neurofilaments

The decrease in neurofilament spacing was reflected in an increased neurofilament density per unit area of the axoplasm. The mean neurofilament densities per 0.0206

μm^2 of the axoplasm in the bin sizes of 0.51-1.00, 1.01-1.50 and 1.51-2.00 μm were increased by 138.6%, 52.9% and 13.3% respectively (table 12). As the decrease in spacing was significant in the first and second groups, an increase in the mean neurofilament density per unit area in these two groups were also statistically significant ($t = 56.35$, $df = 3$, $p < 0.001$) ($t = 6.44$, $df = 2$, $p < 0.05$). There was no significant increase in density per unit area in the third group ($t = 1.83$, $df = 3$, n.s.).

II.dii. Microtubules

The mean density of microtubules per $0.0206 \mu\text{m}^2$ of the axoplasm was decreased from control values by 52.7% in the third group, that in axons of between 1.51 and 2.00 μm diameter. This change was statistically significant ($t = 12.40$, $df = 2$, $p < 0.01$) (table 12). But, the decrease in density of microtubules per unit area in the first and second group by 11.2% and 21.9% respectively, was not statistically significant ($t = 1.77$, $df = 2$, n.s.) ($t = 2.48$, $df = 3$, n.s.)

Table 12

Mean density (\pm SEM) of neurofilaments and microtubules per $0.0206 \mu\text{m}^2$ in axons falling within 0.50 μm wide bins of internodal axonal diameter ranging from 0.51 to 2.00 μm .

	Control			Experimental		
Bin size (μm)	0.51-1.00	1.01-1.50	1.51-2.00	0.51-1.00	1.01-1.50	1.51-2.00
Neurofilaments	3.03 ± 0.06	4.18 ± 0.10	4.28 ± 0.26	7.23 ± 0.04	6.39 ± 0.33	4.85 ± 0.17
Change from control (%)				+ 138.61	+ 52.87	+ 13.32
Microtubules	2.68 ± 0.16	1.55 ± 0.08	1.29 ± 0.05	2.38 ± 0.06	1.21 ± 0.11	0.61 ± 0.02
Change from control (%)				- 11.19	- 21.93	- 52.71

II.e. Changes in the number of neurofilaments and microtubules

II.ei. Neurofilaments

Analysis of changes in the mean number of neurofilaments in complete transverse sections demonstrated that there was an increase in the first and second groups of bin sizes by 38.7% and 19.7% respectively. The change in the first group was significant ($t = 3.87$, $df = 3$, $p < 0.05$), but in the second group was not ($t = 1.59$, $df = 2$, n.s.) (table 13). The increased number of neurofilaments in axons of 0.51-1.00 μm diameter, suggested that the increase in the density per unit area was mainly due to the decrease in their spacing. Thus the increase in density resulted from compaction of neurofilaments, rather than an increase in their number. The mean number of neurofilaments in the third group (with an axonal diameter of 1.51-2.00 μm), however, was decreased from control values by 12%. But this decrease was not statistically significant ($t = 0.50$, $df = 3$, n.s.).

II.eii. Microtubules

There was no significant change in the number of microtubules (table 13) in any of the three axonal bin sizes ($t = 0.91$, $df = 2$, n.s.) ($t = 0.92$, $df = 3$, n.s.) ($t = 2.59$, $df = 3$, n.s.).

Table 13

Mean number (\pm SEM) of neurofilaments and microtubules in axons falling within 0.50 μm wide bins of internodal axonal diameter ranging from 0.51 to 2.00 μm .

	Control			Experimental		
Bin size (μm)	0.51-1.00	1.01-1.5	1.51-2.00	0.51-1.00	1.01-1.50	1.51-2.00
Neurofilaments	31.57 \pm 2.5	75.1 \pm 7.4	173.3 \pm 34	43.8 \pm 1.9	89.87 \pm 1.6	152.5 \pm 24
Change from control (%)				+ 38.74	+ 19.67	- 12
Microtubules	24.03 \pm 1.1	36.9 \pm 3.1	68.8 \pm 7.0	20.63 \pm 3.6	30.93 \pm 5.7	43.5 \pm 6.9
Change from control (%)				- 14.15	- 16.18	- 36.77

III. Analysis of Longitudinal Sections

Examination of longitudinal sections of intermediate sized nerve fibres, as estimated by the number of myelin lamellae, once again provided a differential response both between different axons/fibres or along the length of nerve fibres. In longitudinal sections there was either, 1. Compaction of neurofilaments occurred in a proportion of fibres among closely related fibres in which there were no indication of neurofilament compaction (fig. 24), or 2. Linear compacted of neurofilaments (figs. 24-26) occurred over tens of microns of the nerve fibre length (figs. 24 and 25), and was associated with a low number of microtubules (figs. 24-26), or 3. There was a locus of compacted neurofilaments only extending over 4-5 μm in some nerve fibres (fig. 26) associated with disruption or loss of the axolemma, a highly irregular axonal profile and disruption of the myelin sheath (fig. 26), and 4. In the most severely injured fibres myelin lamellae were separated from each other to form intramyelinic spaces (figs. 24 and 25).

Fig. 24. A longitudinal thin section of a number of intermediate sized axons after stretch-injury. Dissociation and loosening of the myelin lamellae and the occurrence of some spaces between them is most pronounced in the first and third fibres from the left. The detailed structure of the axoplasm differs markedly between closely related axons. From left to right, in the first axon, there are almost no microtubules, neurofilaments are linearly in register and the axoplasm is electron lucent. In the second and fourth axons large number of microtubules are present. In the third axon from the left the axoplasm is electron dense and contains longitudinally oriented neurofilaments but there is a notable reduction in the spacing between adjacent neurofilaments. x 44 750.

Fig. 25. A longitudinal thin section of a myelinated axon after stretch-injury. There are some spaces within the myelin sheath. There is separation of the myelin sheath and the axon extending over 8-10 μm of the length of nerve fibre. The linear arrangement of the components of the axonal cytoskeleton is lost. Neurofilaments are disorientated and some microtubules have an oblique orientation. x 53 200.

Fig. 26. A longitudinal thin section of an intermediate sized myelinated axon after stretch-injury. There is focal compaction of neurofilaments and fragmentation (arrowheads) or loss of axolemma (arrows). On the right hand side of the micrograph neurofilaments are absent, instead there is flocculent material. x 42 300.

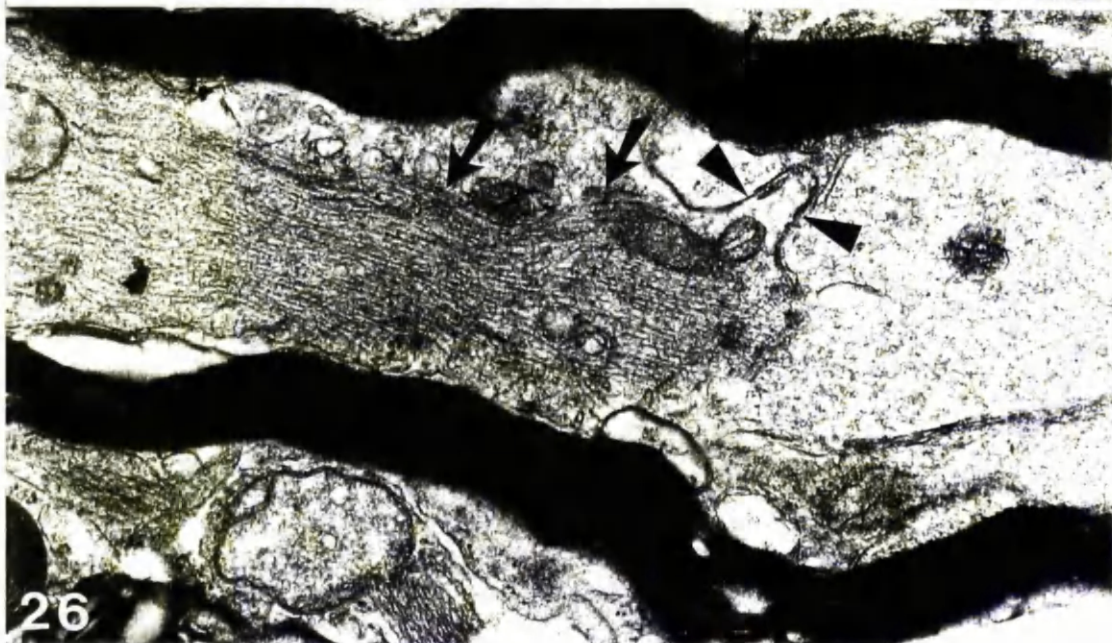
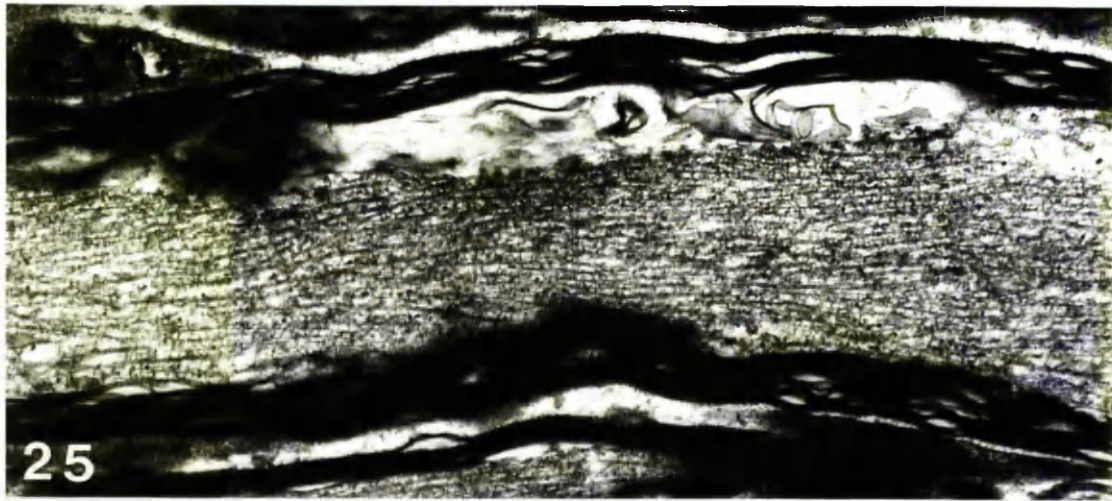
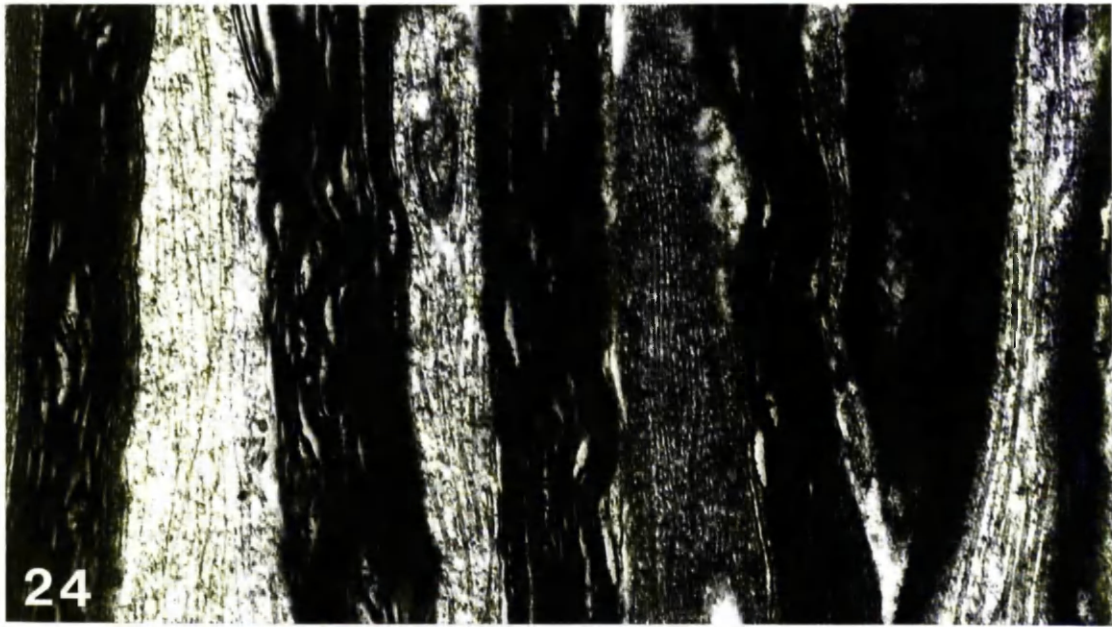
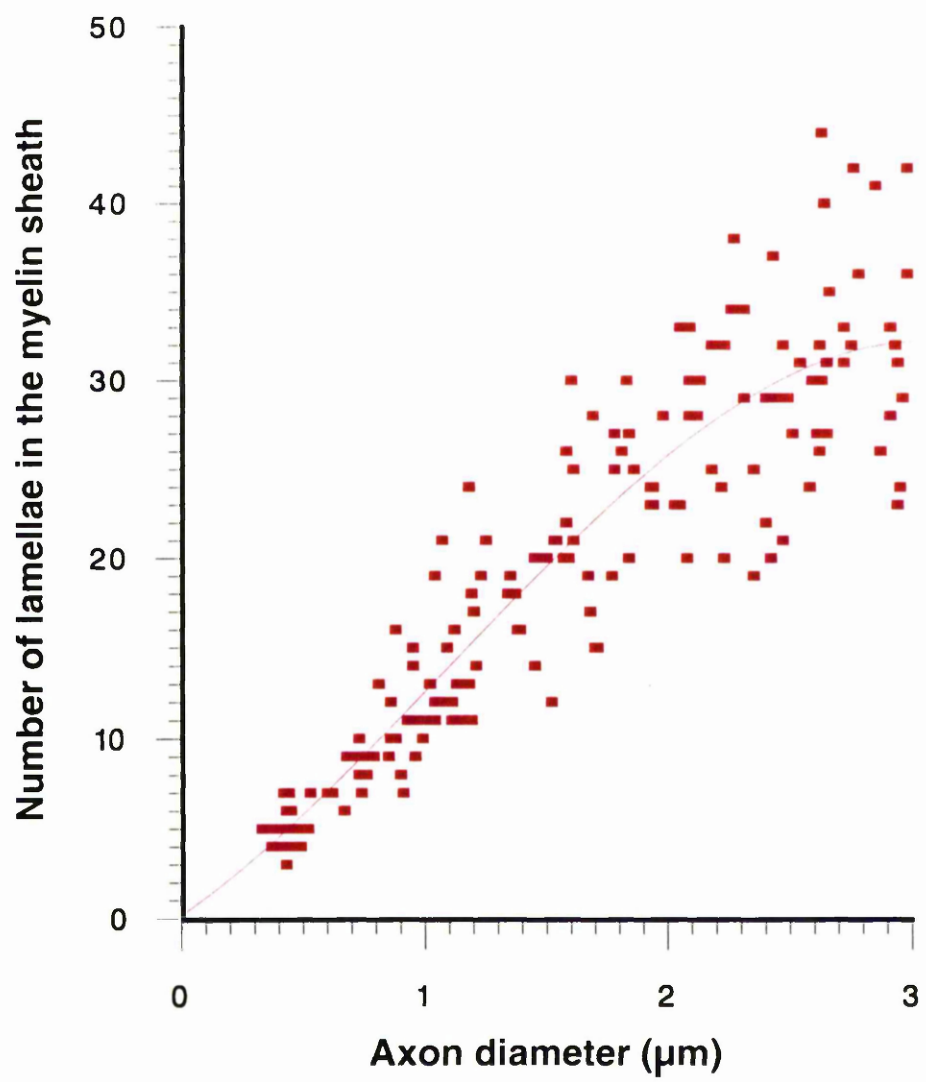


Fig. 27. A graph illustrating the relation between axon diameter and the number of myelin lamellae in nerve fibres from the control adult guinea pig optic nerve (n=3).



CHAPTER SIX

SUMMARY OF PRINCIPAL RESULTS AND DISCUSSION

A. Summary of Principal Results

Until very recently there have been two schools of thought concerning the pathological mechanisms driving or giving rise to the pathology documented (Povlishock, 1992; Maxwell *et al.*, 1997) after non-disruptive axonal injury. These schools have hypothesised that either (i) mechanical tensile strain applied to central, myelinated nerve fibres acted directly upon the cytoskeleton and was manifested as the cytoskeletal changes described by Povlishock and his group (Povlishock, 1992; Christman and Povlishock, 1994). Or (ii) that tensile strain applied to myelinated nerve fibres induced damage to the axolemma of the axon and the compromised function of the axolemma potentiated the development of the observed pathology (Maxwell *et al.*, 1991, 1995, 1997a, b). More recently a consensus has developed between these two schools which has hypothesised that it is in fact damage to the axolemma that is the initiation site for the development of pathology in injured axons (Pettus and Povlishock, 1996; Maxwell *et al.*, 1995 and 1997; Fitzpatrick *et al.*, 1998).

The data presented earlier and summarized below will allow me to provide a better insight as to the pathology and discussion of hypothetical mechanisms operating in non-disruptive axonal injury as occurs in both human DAI (see Introduction) and in traumatic axonal injury (TAI), the now accepted term to refer to axonal changes in animal models of human DAI (Maxwell *et al.*, 1997).

In order to place my own results in context I will, at this point summarize my main findings and compare those findings with published material in a number of models or types of axonal injury (*vide infra*). This may allow me to develop the hypothesis that there is a final common pathway of axonal responses to non-disruptive axonal injury, provide novel evidence that there is a spectrum of axonal pathology in that different

sized axons respond in different ways to non-disruptive axonal injury, provide evidence that microtubules and neurofilaments respond in different ways and with differing time courses to such injury, and that there is a time course over several hours over which such pathology develops and therefore provides the potential for the development of therapeutic interventions to ameliorate such pathology. In the long term it is hoped that the application of such therapies might improve the outcome for the head-injured patient.

Results from the present study

I. Numbers of Axons within Bin Sizes

The greatest degree of axonal and fibre damage occurred in the smallest and the larger fibres of the second segment (middle portion) of stretch-injured optic nerves. The number or proportion of the smallest axons (diameter $< 0.50 \mu\text{m}$) increased by five fold in the second segment and to a lesser degree in the other segments (chapter 3) (table 1; pages 103-104). There is a significant reduction (10.4%) in the number of axons within the bin size of $0.51\text{-}1.00 \mu\text{m}$ in the second segment (table 1; pages 103 and 104) at four hours after injury, and there is no change at the same time point in the number of axons within other bin sizes after stretch-injury.

Following these initial findings a morphometric analysis of changes to the axonal cytoskeleton as seen in transverse sections viz spacing between neurofilaments and between microtubules, density of microtubules and neurofilaments per unit area of

0.0206 μm^2 of the axoplasm and the number of neurofilaments and microtubules in different sizes of axon/fibre diameter were carried out in the second segment.

II. Alterations within the Myelin Sheath and of the Axonal Profile

II.a. Control fibres

In all of control nerve fibres from the smallest to the larger ones the myelin sheath lamellae were uniformly arranged. The profile of the axolemma was smooth and regular, and located close to the myelin sheath (figs. 3, 7a, 18, 20 and 22a).

II.b. Stretch-injured fibres

Irregularity and separation of myelin sheath lamellae from each other to form intramyelinic spaces occurred in almost all sizes of nerve fibres after stretch-injury (figs. 4-6, 8a, 19, 21 and 23a). These spaces were smaller and more focal in smaller fibres (figs. 4-6, 19, 21 and 23a), but were more numerous and wider spread in larger fibres (fig. 8a).

The profile of the axon and axolemma was irregular and in most cases the axolemma lost its close relationship with the myelin sheath (figs. 4-6, 8a, 19, 21 and 23a). In the larger fibres in which periaxonal space(s) occurred the axolemma was separated from the myelin sheath and in most cases the axon was greatly reduced in size (figs. 9a, and 10a).

Periaxonal space(s) occurred throughout the length of stretch-injured optic nerve fibres in 1.3% of fibres in the second segment, but only 0.14% and 0.41% in the first and third segments.

III. Axonal Cytoskeletal Responses

III.a. Numbers of neurofilaments

III.ai. Control axons

The number of neurofilaments in the smallest axons (diameter < 0.50 μm) was less than the number of microtubules (fig. 3). The number of neurofilaments increased with increasing axonal calibre (figs. 3, 7a, b, 18, 20 and 22a, b) where the relation between the number of neurofilaments and axonal diameter was a linear function (slope = 117.57, the correlation coefficient equalling 0.940) (fig. 28). The number of neurofilaments increased from 7.40 ± 1.5 in the smallest axons (diameter < 0.50 μm) to 246.50 ± 9.00 in the larger axons (diameter > 2.00 μm) (chapters 4 and 5) (tables 9 and 13; pages 121 and 164).

This finding extends results obtained from PNS nerve fibres (Friede and Samorajski, 1970; Reles and Friede, 1991) and shows that similar relationships to those already described in PNS fibres apply to optic nerve fibres..

III.iii. Stretch-injured axons

The number of neurofilaments in axons of less than 1.00 μm diameter was significantly greater (figs. 4-6, and 19) than in control axons of the same size (figs. 3 and 18). In the smallest axons (diameter < 0.50 μm) after stretch-injury the number of neurofilaments was higher than the number of microtubules (fig. 31), while in the control fibres of the same size, the number of neurofilaments was lower than the number of microtubules (figs. 28 and 31) (chapter 4) (table 9; page 121).

On the contrary, however, the number of neurofilaments in the axons with a diameter greater than 1.50 μm was decreased from control values (chapters 4 and 5)

(table 9 and 13; pages 121 and 164, figs. 29 and 31), but in the present experimental group (3 animals) this decrease was only significant in the fibres with intramyelinic spaces with an axonal diameter greater than 2.00 μm .

III.b. Microtubule numbers

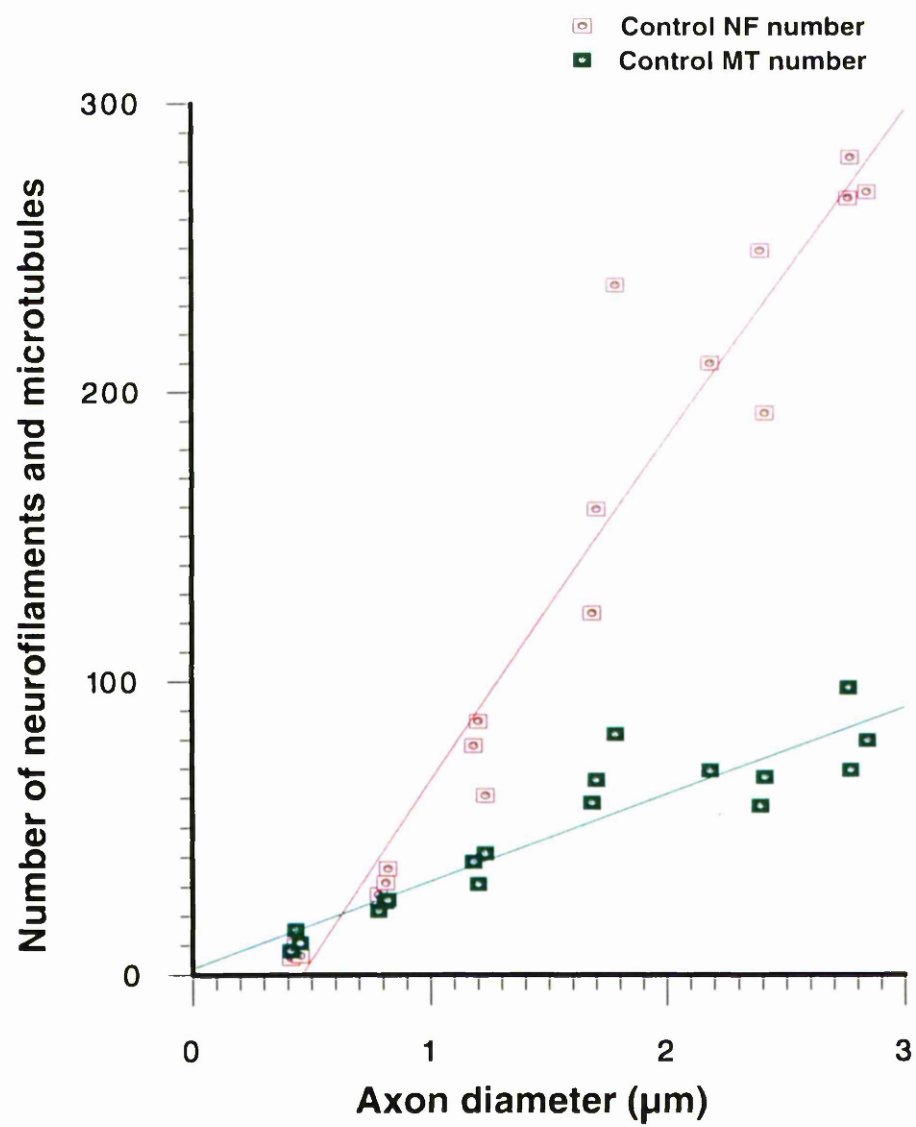
III.bi. Control axons

The number of microtubules increased as the axonal diameter increased. The relation between number of microtubules and axon diameter was again a linear function (slope = 29.68, the correlation coefficient being 0.85) (fig. 28). This novel data from central myelinated axons extends the earlier literature derived from studies in PNS axons (Friede and Somarjski, 1970) that the number of axonal microtubules rises more slowly with increasing axonal diameter than does the number of neurofilaments. The number of microtubules increased from 11.40 ± 2.1 in the small axons (diameter < 0.50 μm) to 74.60 ± 5.5 in the larger axons (diameter > 2.00 μm) (chapters 4 and 5) (tables 9 and 13; pages 121 and 164).

III.bii. Stretch-injured axons

In some examples of the smallest axons (diameter < 0.50 μm) there was a great loss of microtubules (fig. 4). There was no loss of microtubules in axons with a diameter between 0.51-1.00 μm but microtubules tended to be located at the periphery (fig. 19) rather than being uniformly distributed throughout the axoplasm. In the next bin size (1.01-1.50 μm) microtubules were scattered through the axoplasm but with a slightly greater concentration at the periphery (fig. 21). Microtubules in axons with a diameter of between 1.50-2.00 μm were scattered through the axoplasm (fig. 23a). In the

Fig. 28. A graph illustrating the relationships between axonal diameter and the mean number of neurofilaments and the mean number of microtubules per axon of optic nerve fibres from control guinea pigs (n = 3).



stretch-injured larger axons (diameter $> 2.00\ \mu\text{m}$) (fig. 8a) in comparison with control ones (fig. 7a) there was loss of microtubules (figs. 30 and 31)(table 9). A significant loss of the number of microtubules also occurred in fibres with periaxonal space(s) (figs. 9a, and 10a)(table 9).

III.c. Spacing of neurofilaments and microtubules and their density per unit area

III.ci. Neurofilaments in the stretch-injured axons

The neurofilament spacing was significantly decreased from control values in axons with a diameter less than $1.50\ \mu\text{m}$. Therefore, neurofilaments had an increased density and were compacted in these axons after stretch-injury. In some examples from the smallest axons (diameter $< 0.50\ \mu\text{m}$) neurofilaments were compacted and grouped together to form a central core in the axoplasm (fig. 4). A lesser degree of compaction occurred between the smallest (diameter $< 0.50\ \mu\text{m}$) and larger axons with a diameter up to $1.50\ \mu\text{m}$ (figs 19 and 21). Neurofilament compaction did not occur in axons within the bin size of $1.51\text{--}2.00\ \mu\text{m}$ because there was no change in the spacing or density of neurofilaments within their axoplasm (fig. 23a, b). But in the larger fibres (axon diameter $> 2.00\ \mu\text{m}$) there were two different responses. In those fibres with intramyelinic spaces the spacing between neurofilaments increased significantly from control values, or as I have termed it the neurofilaments were “dispersed” (fig 8a,b) (chapters 4 and 5) (tables 7 and 11; pages 120 and 161), and there was a significant loss of the number of neurofilaments. In larger fibres with periaxonal space(s), however, there was compaction rather than “dispersion” of neurofilaments (figs. 9a, b and 10a, b). In these fibres neurofilaments were uniformly distributed, but in a

Fig. 29. A graph illustrating the relationships between axon diameter and the mean number of neurofilaments per axon of control and stretch-injured optic nerves (n=6).

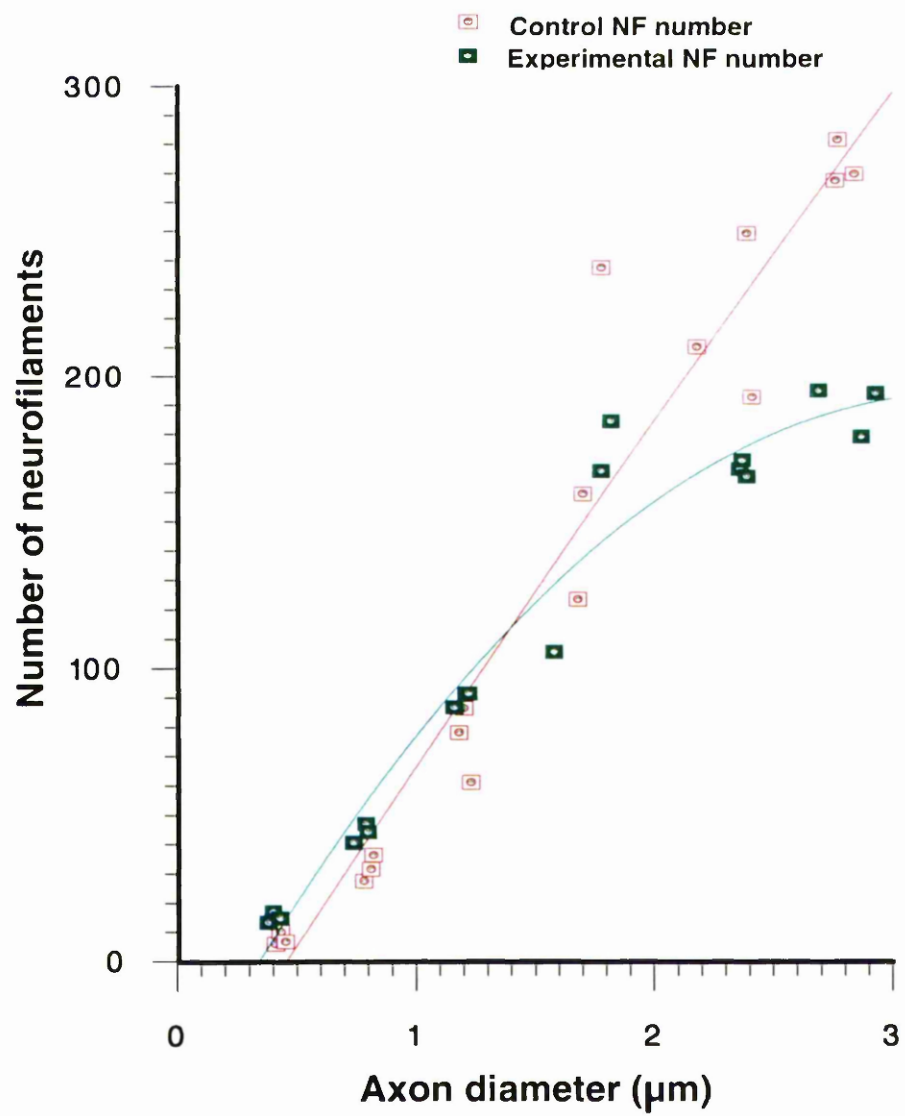


Fig. 30. A graph illustrating the relationships between axon diameter and the mean number of microtubules per axon of control and stretch-injured optic nerve fibres (n=6).

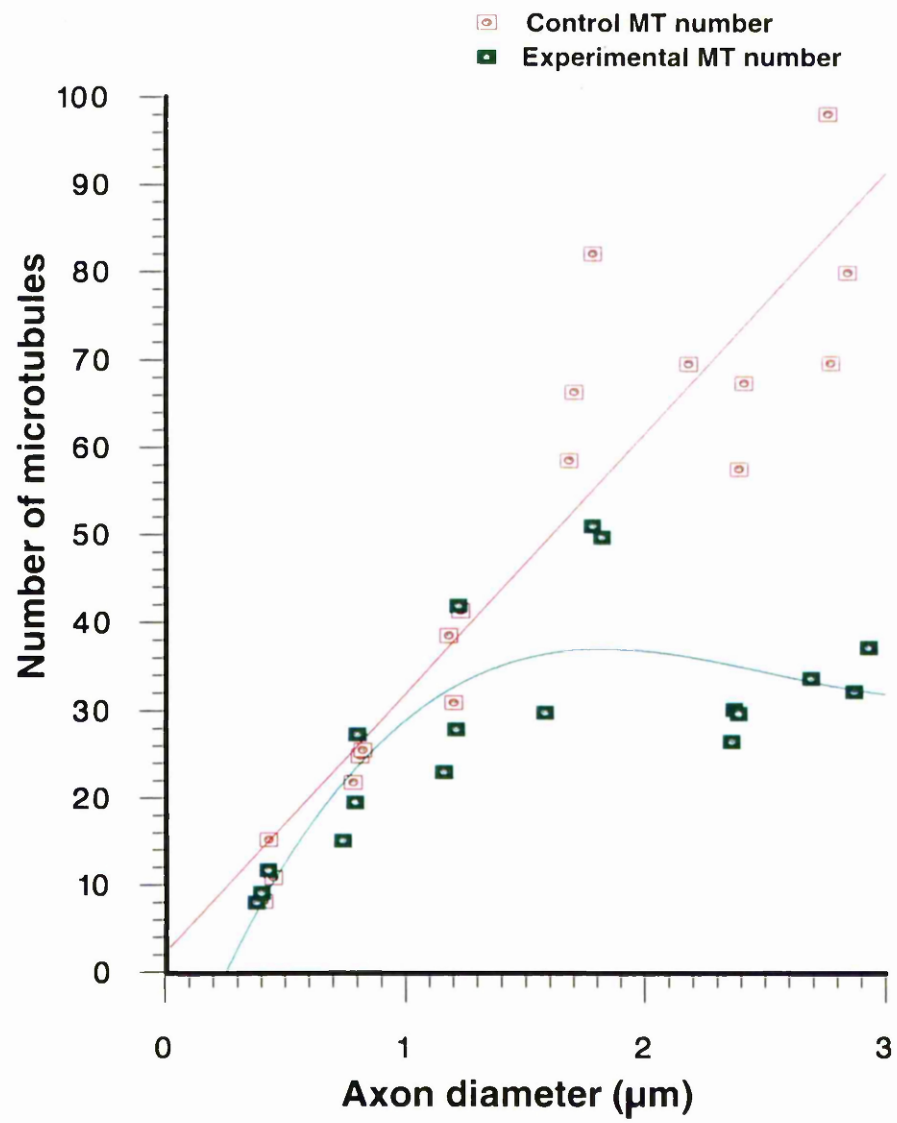
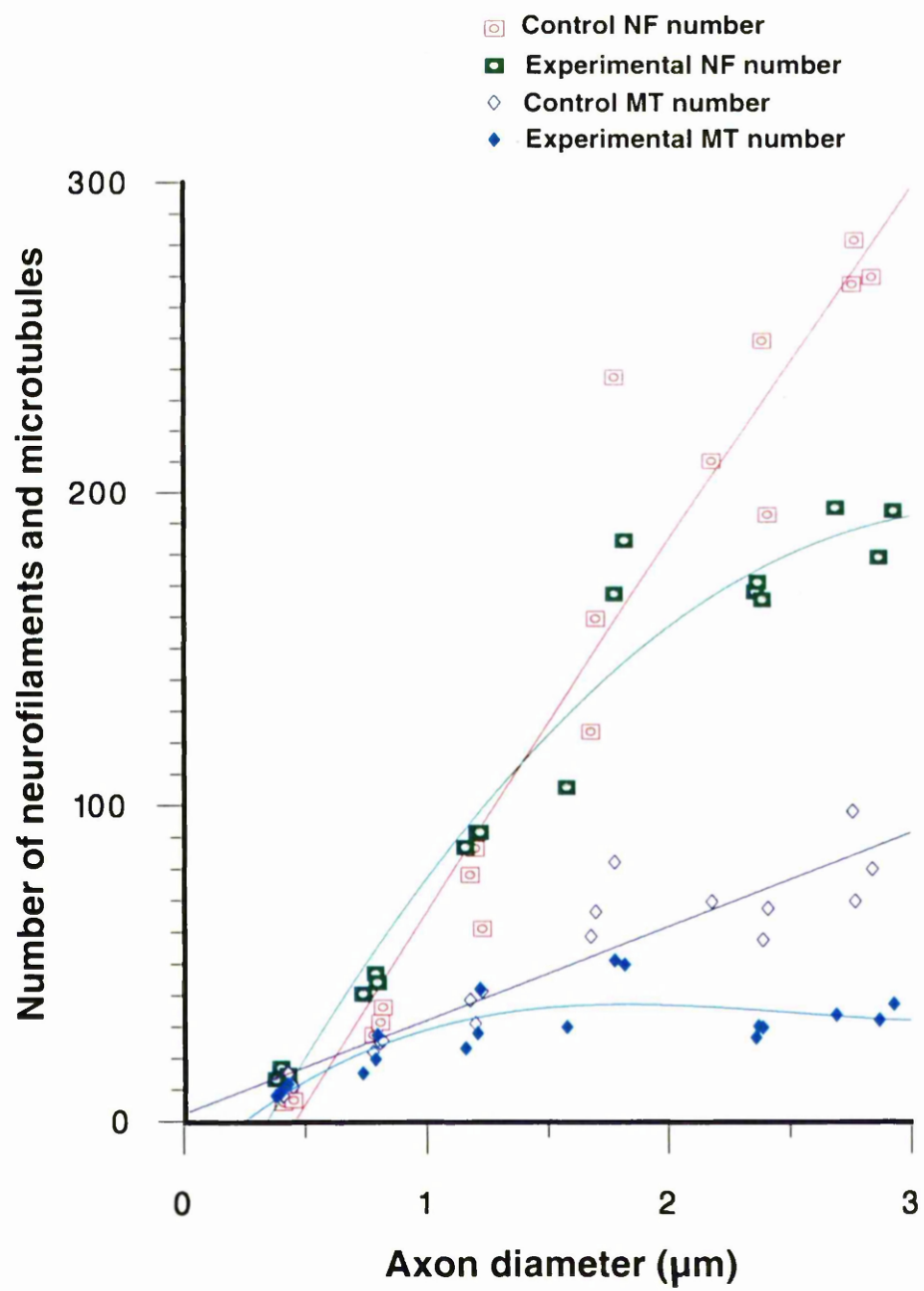


Fig. 31. A graph illustrating the relationships between axon diameter and the mean number of neurofilaments and the mean number of microtubules per axon of control and stretch-injured optic nerves fibres (n=6).



compacted mode, throughout the axoplasm (figs. 9a, b and 10a, b) but there was no change in their number.

The decrease in neurofilament spacing in axons of up to 1.50 μm diameter was associated with a significant increase in the neurofilament density. The density in the axons with a diameter falling within the bin size of 1.51-2.00 μm did not change (chapters 4 and 5) (tables 7, 8, 11 and 12; pages 120, 121, 161 and 162). But in the larger axons with intramyelinic spaces (diameter > 2.00 μm), as the spacing increased, the density decreased significantly from control values. On the contrary, in larger fibres with periaxonal space(s) the spacing decreased significantly (chapter 4) (tables 7 and 8; pages 120 and 121). Therefore, neurofilaments were dispersed in the former but compacted in the latter. The demonstration of post-traumatic dispersion of neurofilaments in larger axons at 4 hours after non-disruptive axonal injury is a novel finding.

III.cii. Microtubules in the stretch-injured axons

A significant increase in microtubule spacing occurred only in axons with a diameter greater than 1.50 μm (chapters 4 and 5) (tables 7 and 11; pages 120 and 161). This increase in microtubule spacing affected the density of microtubules in that the density of microtubules in these axons decreased significantly from control values (chapters 4 and 5) (tables 8 and 12; pages 121 and 162).

Microtubule spacing in larger fibres - diameter greater than 2.00 μm , and with periaxonal space(s) also increased significantly (chapter 4) (table 7; page 120) and there was also a significant decrease in the density of microtubules in these fibres (chapter 4) (table 8; page 121).

The results of the present study, therefore, demonstrated that the response by neurofilaments and microtubules differed after stretch-injury (i) between small and larger fibres and (ii) that responses by these two cytoskeletal components also differed between two subgroups of larger fibres, those with intramyelinic spaces and those with periaxonal space(s). In the fibres with intramyelinic spaces neurofilament spacing was increased and their density was decreased, but in those fibres with periaxonal space(s) neurofilament spacing was decreased (neurofilaments were compacted) and their density was increased. For microtubules, however, no evidence for compaction was obtained and the response by microtubules therefore differed from that of neurofilaments. There was a suggestion of an increased spacing between microtubules in axons with a diameter greater than 0.5 μm but this increase was only real for the experimental group size used in this study ($n=3$) in axons of diameter greater than 1.5 μm . It is worth noting, however, that there was a significant increase in the spacing between microtubules in both subgroups of axons with a diameter greater than 2.0 μm rather than there being different responses as seen for neurofilaments between fibres with intramyelinic spaces and those with periaxonal spaces at 4 hours after injury.

IV. Morphological Analysis of Longitudinal Sections

Morphological analysis of longitudinal sections of optic nerve fibres also provided evidence for novel or previously undescribed pathological changes 4 hours after stretch-injury. Examination of longitudinal sections revealed that: (1) At a suggested mild level of damage where axonal calibre remained uniform (fig. 13), there was a misalignment of the axonal cytoskeleton and in particular of microtubules which adopted a helical disposition rather than lying parallel to the long axis of the axon. (2)

At more severe levels of damage axonal calibre varied to a marked degree over lengths in excess of 10 μm (figs. 14a, b and c). Where axonal calibre was minimal, for example 0.80 μm , (fig. 14a) there was a reduced spacing of neurofilaments and lowered number of microtubules. This was associated with foci of great disruption of the myelin sheath. On the other hand, where axonal calibre was maximal, about 2 μm diameter (fig. 14a), components of the axonal cytoskeleton were misaligned and there was an increased spacing between them. (3) Compaction of neurofilaments occurred in two different groups of axons/fibres, either those axons of less than 1.50 μm diameter or the low number of larger fibres where periaxonal spaces occurred (figs. 12, 15, 16, and 24-26). Compaction of neurofilaments occurred over several microns of the length of smaller nerve fibres (figs. 12, 24 and 25), and in most of the smallest axons compacted neurofilaments grouped together and formed a filamentous central core (fig. 12). In some larger nerve fibres, foci of neurofilament compaction occurred (fig. 15) at sites of increased fibre diameter where the myelin sheath formed “balloon-like” expansions, over about 10 μm of the length of the same fibre. At these sites in the majority of examples there was disruption of the axolemma (fig. 16). Further, these regions were separated by regions of nerve fibre with a normal cytoskeletal structure. (4) Neurofilaments were arranged linearly and parallel both to each other and the long axis of the axon in areas of compaction. In these areas there was also a lower number of microtubules (figs. 12, 16, and 24-26).

B. Discussion

I. Hypotheses

The results summarized above give rise to a number of hypotheses. First I shall list these and then discuss each of them in turn. The hypotheses generated by my work are:

1. That there is clear, morphometric evidence for a spectrum of axonal pathology after traumatic axonal injury in that different sized axons demonstrate a different pathology.
2. That there are differences between the pathology of the axonal cytoskeleton after traumatic axonal injury and the pathology documented in other types or models of axonal injury; for example, transection or crush injury and primary axotomy.
3. That this work questions the hypothesis in the current literature relating to traumatic axonal injury that neurofilament responses to such injury are of one type.
4. That there are differences in the pathology or response between neurofilaments and microtubules after traumatic axonal injury.

- 1. That there is clear, morphometric evidence for a spectrum of axonal pathology after traumatic axonal injury in that different sized axons demonstrate a different pathology.**

That there is a spectrum of axonal pathology after TAI was first hypothesised by Povlishock in 1992. He suggested that “Paralleling important studies of severe traumatic injury in both animals and Man, studies conducted in humans who had sustained only mild or moderate traumatic brain injury also identified the presence of damaged axons. Although reduced in both number and overall anatomical distribution

in comparison to the severe traumatic state, their very occurrence with mild and moderate injury suggested that axonal damage occurs across the spectrum of traumatic injury.”

More recent results obtained from the study of human head-injured patients and the use of now accepted more sensitive markers of axonal injury have supported the hypothesis given above. Use of the β -amyloid precursor protein (β -APP) immunostaining technique for detection of damaged axons in human fatal head injury (Sherriff *et al.*, 1994; Blumbergs *et al.*, 1994, 1995; Gentleman *et al.*, 1995; McKenzie *et al.*, 1996) has been shown that the occurrence of axonal damage is most frequent in the brain stem followed by internal capsule, thalamus, corpus callosum and parasagittal white matter of human brains after fatal head injury (McKenzie *et al.*, 1996). Immunolabelling of (β -APP) has also revealed that axonal damage in humans is far more widespread than it was thought previously and in fact axonal injury may be the most common result of human fatal head injury (Gentleman *et al.*, 1995). Use of this technique has shown that morphologically damaged axons occur as early as 1.75-2 hours after injury (Blumbergs *et al.*, 1995; McKenzie *et al.*, 1996) and are still present as long as 99 days after mild head injury in humans (Blumbergs *et al.*, 1994). There is now a consensus (Maxwell *et al.*, 1997b) that in human diffuse axonal injury primary axotomy, that is tearing or breakage of the axons at time of injury, does not occur in the majority of axons but rather they undergo a non-disruptive axonal injury (Maxwell *et al.*, 1993, 1997b) in which a series of reactive axonal changes (Povlishock *et al.*, 1983; Povlishock, 1992) leads to disruption/disconnection of axons over at least several hours that is now termed secondary axotomy (Maxwell *et al.*, 1993). In secondary axotomy disconnection may occur, depending upon species, any time

between 1 and 6 hours in animal models (Erb and Povlishock, 1988) and 12 hours after injury in humans (Blumberges *et al.*, 1994, 1995; Christman *et al.*, 1994; Grady *et al.*, 1993) (see also pages 44-45).

Experimental support from animal models for the hypothesis that there is a spectrum of axonal pathology has also been provided by later work (Pettus *et al.*, 1994; Pettus and Povlishock, 1996) in that these authors subdivided pathological changes in the axonal cytoskeleton after fluid percussion injury in the cat into (a) mild levels of injury resulting in blebbing or infolding of the axon, misalignment of the cytoskeleton, increased immunolabelling for the 68 kDa neurofilament subunit and swelling of axons between 30 min and 2 - 3 hours after injury when axotomy occurred; and (b) moderate levels of injury where there was microvacuolation of the axoplasm, a permeabilisation of the axolemma to horseradish peroxidase (HRP) and a reduced spacing between neurofilaments from 45.46 nm in controls to 14.95 nm in injured axons within minutes of injury.

Thus, in conclusion, the hypothesis that there is a spectrum of pathology was based upon the concept that some axons were more severely injured than others at the time of injury (Povlishock, 1992; Pettus *et al.*, 1994; Pettus and Povlishock, 1996) and that more severely injured axons progressed more rapidly along the path of reactive axonal change leading to secondary axotomy.

The results presented in the current study provide clear evidence that the pathology is more complex than Povlishock and his group had earlier envisaged in that there is now good, quantitative evidence that different sorts of damage to the axonal cytoskeleton occur in different sizes of axon; that compaction of neurofilaments occurs in axons of 1.00 μm or less diameter, that dispersion of neurofilaments occurs in axons

of 2.00 μm or greater diameter in which intramyelinic spaces occur in the myelin sheath and that compaction also occurs in axons of 2.00 μm or greater diameter where periaxonal spaces are present after injury. A direct correlation between the results presented here and those presented by Pettus *et al* (1994) and Pettus and Povlishock (1996) cannot, at this time be made because evidence for a permeabilisation of the axolemma in the guinea-pig optic nerve model has not been provided. However, this model has provided cytochemical evidence which supports the concept of damage to the axolemma in that there are changes in the distribution of axolemma membrane pumps (Maxwell *et al.*, 1995) or quantitative freeze-fracture evidence for alterations in the structure of the axolemma (Maxwell, 1996; Maxwell *et al.*, 1998) after stretch-injury. These latter studies in particular indicate damage to the axolemma which is (i) reflected in a loss of its control of intra-axonal ionic homeostasis and (ii) extends over days after injury. A direct correlation with the studies of Povlishock's group can only be made once it can be demonstrated in the guinea-pig optic nerve that the axolemma is indeed permeable to tracers after injury. It is worth noting at this point that it may be suggested that the use of markers as large as HRP (Pettus *et al.*, 1994; Pettus and Povlishock, 1996) may not be appropriate and it would be better to use markers of ionic rather than macromolecular proportions, for example lanthanum nitrate.

Nonetheless, the results presented in the current study do clearly demonstrate that smaller and large axons react to stretch-injury in different ways in that the changes occurring in the axonal cytoskeleton at 4 hours after injury are clearly different. Consideration of the mechanisms resulting in these changes in the structure of the axonal cytoskeleton must, at this time, be hypothetical (*vide infra*). But the results clearly suggest that there is now the possibility that the differences in response by

different sized axons may reflect differences in the detailed biochemistry of the axonal cytoskeleton between different sized axons. This type of study will necessitate the use of immunocytochemical techniques utilising antibodies between different subunits of the axonal cytoskeleton and, further, a consideration of the biochemical mechanisms which serve to maintain the structure and integrity of the normal axonal cytoskeleton. There is some evidence for such changes at present (Yaghmai and Povlishock, 1992; Christman *et al.*, 1994; Pettus *et al.*, 1994) but this data relates specifically to changes in the level of labelling principally for the 68 kDa neurofilament subunit. A more detailed analysis of changes in the biochemistry of the interaction between neurofilaments, and microtubules (*vide infra*) would therefore be of very considerable interest at this time.

2. That there are differences between the pathology of the axonal cytoskeleton after traumatic axonal injury and the pathology documented in other types or models of axonal injury; for example, transection or crush injury and primary axotomy.

Documented changes in the axonal cytoskeleton in Wallerian degeneration have, with one recent exception, only been investigated at least 24 hours after axonal injury and then only in the PNS, for example between 24 and 48 hours after transection of the rat sural nerve (Ballin and Thomas, 1969), in the sciatic nerve of rats, rabbits and cats (Martinez and Friede 1970; Donat and Wisniewski, 1973; Schlaepfer and Micko, 1978), in the saphenous nerve of the rat (Schlaepfer, 1974) and after crush injury to the rat sciatic nerve (Hoffman *et al.*, 1985b). Loss of neurofilaments and microtubules and their replacement with an accumulation of loose floccular material has been

observed up to several centimetres from the site of transection in rabbit and cat sciatic nerves (Donat and Wisniewski, 1973). In a more recent study (McHale *et al.*, 1994; Hall and Lee, 1995), however, densely packed neurofilaments forming so called 'condensed filamentous cores' have been observed in the first few hours after transection of giant axons in the spinal cord of the lamprey. The condensed neurofilamentous cores possessed an ultrastructure which demonstrated considerable parallels with foci of neurofilament compaction seen in axons at 4 hours after stretch-injury to the guinea-pig optic nerve. The observations of McHale *et al.*, (1994) and Hall and Lee (1995) have provided the first evidence for compaction of neurofilaments in the early stages of Wallerian degeneration. In this context it is worth noting that these condensed filamentous cores were only located in the "lesion" site, that is in the proximal and distal stumps closest to the site of transection of the axon. Use of Western blots (Hall and Lee, 1995) provided evidence that the occurrence of condensed neurofilamentous cores was correlated with proteolysis of neurofilament sidearms and immunocytochemical evidence strongly suggested that neurofilaments within the condensed cores were dephosphorylated. However, evidence for such changes in neurofilament spacing or phosphorylation of sidearms at sites of axonal transection in studies of early changes during Wallerian degeneration in mammals is still lacking.

Morphological evidence for rapid and complete loss of the axonal cytoskeleton and its replacement with a flocculant ultrastructure has only been provided in one model of human DAI, the non-human primate head acceleration model. At severe levels of injury in that model evidence for tearing or shearing of axons has been provided (Maxwell *et al.*, 1993). In this study, evidence for the occurrence of large holes or foci

of discontinuity, termed fragmentation, of the axolemma was provided between 20 - 35 minutes after lateral acceleration of the head. Further, at the sites where the axolemma was fragmented the axoplasmic cytoskeleton was no longer recognisable but, rather, was replaced by a flocculent ultrastructure which was interpreted as morphological evidence for proteolysis of the cytoskeleton (Maxwell *et al.*, 1993). Comparable evidence has not been provided in any other model of TAI or human DAI (reviewed by Maxwell *et al.*, 1997b). The results obtained in the present study therefore provide further evidence that there is not rapid and acute loss of axonal cytoskeletal structure at the point of injury but rather a time course of changes extending over at least several hours after injury, 4 hours in the case of the present study and up to 6 hours after fluid percussion injury in the cat (Pettus and Povlishock, 1996) in which there is rapid and long lasting re-organisation of the axonal cytoskeleton with the retention of a filamentous form of the axonal cytoskeleton.

In the present study, despite loss of neurofilaments in fibres with intramyelinic spaces (axonal diameter greater than 2.00 μm) (table 9; page 121) (Jafari *et al.*, 1997), retention of neurofilaments occurred in a large number of axons with a diameter less than 2.00 μm (table 13; page 164). These findings confirm and extend those of several other studies where retention of the filamentous form of neurofilaments occurs over at least a number of hours after TAI, for example:- 5 minutes to 5 hours after moderate and 6 hours after moderate to severe fluid-percussion traumatic brain injury in cats (Pettus *et al.*, 1994; Pettus and Povlishock, 1996; Povlishock and Pettus, 1996), 2 hours after impact acceleration to rat skulls (Povlishock *et al.*, 1997) and one hour after moderate traumatic brain injury in cats and rats (Yaghmai and Povlishock, 1992). Thus evidence for a rapid loss of neurofilaments after TAI has not been obtained.

In the fluid percussion (Pettus and Povlishock, 1996) and cortical impact (Povlishock *et al.*, 1997) models of TAI compaction or close-packing of neurofilaments has been demonstrated over considerable, tens of micrometers, lengths of injured axons. But no evidence for foci of compaction of neurofilaments between sites where the structure of the axonal cytoskeleton is normal within the same axon has been provided. Neither has evidence that compaction of the neurofilamentous cytoskeleton is correlated with disruption of the integrity of the axolemma been provided directly. Indeed Pettus and Povlishock (1996) state that changes in the organisation of the neurofilamentous cytoskeleton occur within minutes of injury and remain unchanged over the ensuing 4-6 hours. Thus the present findings of sites of focal compaction of neurofilaments associated with disruption of the axolemma are novel. Further, the finding in the present study of loss of the integrity of the axolemma related to these sites of neurofilament compaction provide what can be, at least, suggested to be the first morphological evidence for the process of secondary axotomy occurring hours after injury.

3. That this work questions the hypothesis in the current literature relating to traumatic axonal injury that neurofilament responses to such injury are of one type.

The early literature relating to studies of either human head injury or models thereof (see Introduction; Nevin, 1967; Peerless and Rewcastle, 1967; Strich, 1970) hypothesised that axons were sheared at the time of injury. Only one animal model, lateral acceleration of the non-human primate head (Gennarelli *et al.*, 1982; Maxwell *et al.*, 1993) has provided support for this hypothesis. Rather, there is now a

consensus that in all but the severest forms of human head injury, axons are not sheared at the time of injury but rather injury to the axolemma initiates a “pathological cascade” of events resulting in secondary axotomy at least several hours after injury (Maxwell *et al.*, 1997b). The results of the present study extend and support these latter findings in that neurofilaments remain within injured axons for several hours after injury and there is not dissolution of the neurofilamentous cytoskeleton in injured axons as would occur in primary axotomy. Rather there are changes in the detailed structure and relationships of the neurofilamentous cytoskeleton (Povlishock *et al.*, 1983; Yaghai and Povlishock 1992; Povlishock, 1992; Christman *et al.*, 1994; Pettus *et al.*, 1994; Pettus and Povlishock, 1996; Povlishock and Pettus, 1996; Jafari *et al.*, 1997; Povlishock *et al.*, 1997) in that there is a single, discrete response by neurofilaments after TAI, that of neurofilament compaction. Compaction of neurofilaments occurs within minutes of injury and the spacing between neurofilaments thereafter is unchanged over the next several hours (Pettus *et al.*, 1994; Pettus and Povlishock, 1996). From this literature it may be concluded that neurofilaments respond to the application of tensile strain to axons by becoming compacted within the axoplasm. However, the results of the present study do not fully support this finding in that in axons with a diameter of more than 2.00 μm in the stretch-injured guinea pig optic nerve at 4 hours there was novel evidence for “dispersion” of neurofilaments in axons in which intramyelinic spaces occurred in the myelin sheath. Novel data in the present study extends previous work by demonstrating that rather than just a single response to injury in the form of compaction of neurofilaments in some axons, specifically larger axons of the optic nerve, neurofilament numbers may be reduced and the spacing between neurofilaments increased post-trauma. This is a novel and

unique finding which clearly demonstrates that there is more than one response by neurofilaments after TAI and now means that in any future analysis of axonal responses in models of human head injury increased spacing between neurofilaments in axons in close spatial relationship to those demonstrating compaction may reflect the fact that those axons may be damaged. This novel finding also raises another important question as to what is the long term outcome for axons in which neurofilament dispersion has occurred after TAI.

4. That there are differences in the pathology or responses between neurofilaments and microtubules after traumatic axonal injury.

Neurofilaments provide the structural cytoskeleton of the axon (Burgoyne 1991) and changes in their number and the cross-linking between adjacent neurofilaments occurs both in development (Nixon *et al.*, 1994) and the state of myelination of the axons (Nixon *et al.*, 1994; de Waegh *et al.*, 1992). Microtubules, on the other hand, are the conduits for the process of fast axonal transport (Burgoyne 1991).

The present study has provided clear, quantitative, morphometric evidence that there are differences in the number of microtubules in the internode of axons of 2.0 μm or greater diameter between control and stretch-injured axons. This study also provides the first clear evidence that whereas the changes in spacing between neurofilaments differs between axons of different size that in the larger fibres of the optic nerve there is a reduction in the number and a decrease in the density of microtubules whether neurofilament spacing is reduced or increased after stretch-injury. Thus the same change for microtubules occurs in axons despite the fact that responses by neurofilaments differ between groups of axons. These results extend

recent studies by Maxwell and Graham (1997) and allow the drawing of the conclusion that the time course of microtubular responses to stretch-injury differs from that of neurofilaments. At 15 min after stretch-injury there is a dramatic loss of microtubules, to 25 % of the control number in the internode (Maxwell and Graham, 1997). A similar reduction in the density of microtubules, by 77%, shortly after fluid percussion injury has also been documented by Pettus and Povlishock (1996). But those latter authors provide no other information with regard to changes with respect to microtubules for either different sized axons or whether there is any time course for these changes. A novel finding in the present study is that at 4 hours the only axons in which there is still a reduction in the number of microtubules at the internode is in the larger fibres in the guinea-pig optic nerve. That is to say that the numbers of microtubules in the internode of axons of less than 2.00 μm diameter returns to normal or control values within 4 hours of injury in axons in which their circularity was greater than 0.8. One interpretation of this change is that there is a return of microtubules within axons between injury and 4 hours. Other evidence in support of this hypothesis is provided by Maxwell and Graham (1997). Clearly, however, rigorous evidence for recovery of microtubules over a matter of hours after injury is urgently required. But it may presently be hypothesised that after stretch-injury microtubules acutely disappear from injured axons and repolymerise over a few hours after injury. Loss of microtubules provides a straightforward explanation for loss of fast axonal transport (FAT) but also implies that, if microtubules do recover after injury, that axons may resume normal modes of FAT. Maxwell and Graham (1997) showed that in axonal swellings characterised by aggregates of membranous organelles numbers of microtubules remained low at 2 and 4 hours after injury. It may therefore

be hypothesised that where microtubules return toward control values after injury the axons recovers. But where that does not occur the axon then proceeds to the formation of one or more axonal swellings and secondary axotomy.

Further evidence in support of the hypothesis that microtubules and neurofilaments respond differently to stretch-injury is provided by the fact that in axons of 1.00 μm or less diameter the number of neurofilaments is significantly increased at 4 hours after injury. But the number of microtubules does not differ from control values. But, as indicated above, there is loss of microtubules at 4 hours in axons of diameter greater than 2.00 μm . Thus responses by microtubules to stretch-injury also differ between different sizes of axon.

The results of the present study, together with other data generated using the optic nerve stretch-injury model, provide the first quantitative, morphometric evidence that (i) the time course of the response by microtubules in TAI differs from that of neurofilaments, (ii) that there is a different response by microtubules between small and larger axons and (iii) that there may be recovery of microtubules in axons over the first several hours after injury. The latter situation clearly provides opportunity for the testing of the effectiveness of therapies to ameliorate the effect of injury and the development of axonal pathology.

II. Hypothetical Pathological Mechanisms in the Changes of the Organisation and Relations of the Axonal Cytoskeleton

II.a. Reduction in the spacing between neurofilaments and compaction

It has been hypothesised that proteolysis of neurofilament side-arms causes neurofilament compaction (Hall and Lee, 1995; Pettus and Povlishock, 1996;

Povlishock and Pettus, 1996). More recently, it has been hypothesised that the collapse or loss of neurofilament side-arms may occur through either/or in combination (1) proteolysis of neurofilament side-arms by increase in intra-axonal Ca^{2+} concentration and activation of μM calpain and (2) dephosphorylation of neurofilament side-arms (Maxwell *et al.*, 1997b).

(1) Proteolysis of neurofilament side-arms by increase in intra-axonal Ca^{2+} concentration and activation of calpains:-

Adams *et al.*, (1991) suggested that tensile strain may damage the axolemma and as a result membrane channels open at the time of injury and provide a mechanism for an hypothesised, uncontrolled influx of calcium. It has been also suggested that mechanoporation is the most probable mechanism in initiating abnormalities in brain injury through the production of traumatic cell membrane defects which provide a transient separation of the cell lipid bilayer from protein inclusions or channels in the membrane (Gennarelli, 1997). Hypothetically, this provides for great and rapid influx or efflux of ions depending on the pre-injury concentration gradients (Gennarelli, 1997). Further, cytochemical evidence for a loss of membrane pump Ca^{2+} -ATPase activities at nodes of Ranvier 15 min to 6 hours and precipitation of pyroantimonate which identifies areas of calcium influx at nodal blebs at the node of Ranvier, swelling of axonal mitochondria and loss of ecto-Ca-ATPase activity from the myelin sheath (Maxwell *et al.*, 1995, 1997b) provides evidence for alteration in membrane function after stretch-injury. Thus, toxic levels of free ions may occur in the axoplasm as the results of either alteration in the structure of the membrane so that it becomes dysfunctional (Maxwell *et al.*, 1997a) or there is a change in axolemmal permeability (Pettus and Povlishock, 1996).

It has been shown that proteolytic activity of neutral proteases, known as calpains, on neurofilament triplet proteins, tubulin and microtubule associated proteins (MAP) is stimulated by increased calcium concentration (Ishizaki *et al.*, 1983; Kamakura *et al.*, 1985; Billger *et al.*, 1988; Cottin *et al.*, 1991; Banay-Scwartz *et al.*, 1994). But there is a requirement for different concentrations of calcium to allow proteolytic breakdown of neurofilaments and microtubules, the latter being much more sensitive (Job *et al.*, 1981). Two calpains, mM calpain and μ M calpain found in axons (Job *et al.*, 1981; Kamakura *et al.*, 1985; Billger *et al.*, 1988; Cottin *et al.*, 1991) degrade neuronal cytoskeletal proteins such as MAP2, actin, spectrin and neurofilaments (Murachi, 1989). The mM calpain has a low affinity for calcium and requires at least 0.20 mM calcium for activation (Glass *et al.*, 1994), while μ M calpain has a high affinity for calcium and is activated at less than 0.1 mM Ca^{2+} (Kamakura *et al.*, 1985; Schlaepfer and Zimmerman, 1985). It has been demonstrated that μ M calpain is more specific for targeting its substrate and it has been found that neurofilament side-arms are the primary target (Ishizaki *et al.*, 1983; Kamakura *et al.*, 1985). Proteolysis of neurofilament side-arms, 200 kDa (NF-H), has been shown to occur 12 hours after axotomy of lamprey spinal cord axons (Hall and Lee, 1995). This side-arm proteolysis was associated with clear labelling by rod monoclonal antibodies corresponding to densely packed neurofilaments forming a condensed neurofilamentous core or mass (Hall and Lee, 1995). Hall and Lee (1995) suggested that the molecular properties of neurofilaments were greatly affected by the proteolysis of neurofilament side-arms such that neurofilaments appeared to be resistant to further proteolysis up to 3 weeks postinjury in the distal stump (Hall and Lee, 1995). Since neurofilaments in lamprey are monopolymers of 180 kDa subunits (Pleasure *et al.*, 1989; Hall and Lee, 1995),

the post-injury changes may not be directly comparable with those in the heteropolymeric neurofilament triplet protein in mammalian axons. The differences between mammalian neurofilaments and those in the lamprey also may account for the temporal differences in neurofilament compaction. But in spite of the differences between mammalian and lamprey neurofilaments, Pleasure *et al* (1989) concluded that lamprey neurofilaments show essential features of mammalian neurofilaments such as a filament forming core and a carboxy-terminal extension with multiple phosphorylation sites. Povlishock and colleagues (1997) also believe that both changes, in respect of neurofilament side-arm loss/or alteration and compaction of neurofilaments, in lamprey and in rodents appeared likely to be identical except for the time course of changes that is longer in the lamprey.

Activation of μ M calpain through elevation of intra-axonal levels of calcium are therefore hypothesised to result in removal or loss of neurofilament side-arms. This loss of side-arms is manifested ultrastructurally as compaction of neurofilaments.

(2). alteration in the state of neurofilament side-arm phosphorylation

There is now a considerable literature relating to the effect/importance of phosphorylation of neurofilaments, particularly NF-H and to a lesser extent NF-M, in the modulation of neurofilament core and side-arm structure. Early work suggested that phosphorylation of neurofilaments stabilises their structure (Pant, 1988).

It is well known that the neurofilament side-arms, in particular the carboxy-terminal tail domains of NF-M and more particularly NF-H, are involved in interaction between neurofilaments (Nixon and Sihag, 1991; de Waegh *et al.*, 1992; Brady, 1993; Nixon *et al.*, 1994). In normal axons the spacing between neurofilaments is mostly controlled by NF-H phosphorylation, whereby an increase/decrease in NF-H phosphorylation is

correlated with an increase/decrease in interneurofilament spacing (de Waegh *et al.*, 1992; Mata *et al.*, 1992; Hsieh *et al.*, 1994; Tu *et al.*, 1995). *In vivo* studies on the intact optic nerve of the mouse have revealed that the minimum and average distance between neurofilaments is greatly increased by phosphorylation of the carboxy terminus domain of NF-H and NF-M (Nixon *et al.*, 1994). In more recent transgenic experiments genes for human NF-M were transfected into one cell stage embryos of mice and resulted in an increase in the level of NF-L protein and reduced content of the phosphorylated isoform of NF-H in the brain of transgenic mice which, in turn, was correlated with an increase in the density of neurofilaments within large-diameter CNS axons in the transgenic compared to the wild-type (Tu *et al.*, 1995). These results, together with those obtained by de Waegh and colleagues (1992), strongly suggest that where neurofilament spacing is reduced there is a fall in the level or degree of phosphorylation of neurofilament side-arms (Tu *et al.*, 1995). Tu and colleagues (1995) concluded that NF-H which has more potential sites in its carboxy-terminal domain for phosphorylation, and to a lesser extent NF-M, are the major, if not only, regulators of neurofilament spacing and that their phosphorylation state is regulated in an organised and controlled way. It is now established that phosphorylation causes an increase in side-arm extension and consequently an increase in interneurofilament spacing (Nixon and Sihag, 1991; de Waegh *et al.*, 1992; Brady, 1993; Hsieh *et al.*, 1994; Nixon *et al.*, 1994; Tu *et al.*, 1995). The negative charges that are associated with phosphorylation sites on the carboxy terminal side-arms, increase the repellent forces which control interneurofilament spacing (Nixon and Sihag, 1991; de Waegh *et al.*, 1992; Brady, 1993; Nixon *et al.*, 1994; Tu *et al.*, 1995). But when dephosphorylated the side-arms have little charge which allows the core

filament to close the space (de Waegh *et al.*, 1992).

Thus, it may be hypothesised that dephosphorylation would result in or contribute to a reduction in interneurofilament spacing and a correlated compaction of neurofilaments. Evidence to support this hypothesis has been obtained from the myelin deficient Trembler mouse where a reduced degree of phosphorylation of heavily phosphorylated NF-H and an increased content of dephosphorylated NF-H was correlated with an increase in packing density of neurofilaments (de Waegh *et al.*, 1992).

Pant (1988) suggested that phosphorylation of neurofilaments protected neurofilaments against proteolysis. Pant (1988) demonstrated that in isolated bovine spinal cord, neurofilaments which were dephosphorylated by alkaline phosphatase were more extensively degraded and at a higher rate in the presence of calpain and CaCl_2 than phosphorylated neurofilaments. Further NF-H was degraded more significantly, more rapidly and to a greater degree after dephosphorylation (Pant, 1988). More recent evidence in support of this hypothesis was provided by Posmantur *et al.*, (1996) who found 3 hours after traumatic brain injury in rats, various degrees of NF-H disassembly by confocal electron microscopy in areas of neurofilament fragmentation, and, significantly, to a subtle and lesser degree in NF-L in dendrites in a light microscopic examination using immunofluorescence techniques. Posmantur and colleagues (1996) suggested that protein degradation may be regulated by the level of phosphorylation of NF-H since, in dendrites in comparison to axons NF-H is largely unphosphorylated (Gotow and Tanaka, 1994) and therefore susceptible to proteolysis. Phosphorylated 200 kDa neurofilament protein which was previously dephosphorylated by incubation with phosphatase was degraded by the activation of

calcium-independent endogenous proteinase (Goldstein *et al.*, 1987). Degradation of NF-H did not occur in the presence of a phosphatase inhibitor. It was therefore suggested that phosphorylation protected neurofilaments from proteolysis.

Side-arm loss also have been observed after fluid-percussion brain injury in cats (Pettus and Povlishock, 1996; Povlishock and Pettus 1996) and impact acceleration of the skull in rats (Povlishock *et al.*, 1997). Since carboxy-terminal tail (side-arms) domains in medium and particularly high molecular weight neurofilament subunits hold the spacing between neurofilaments (Carden *et al.*, 1987; Julin *et al.*, 1988; Lee *et al.*, 1988; Nixon and Sihag, 1991; de Waegh *et al.*, 1992; Mata *et al.*, 1992; Brady, 1993, Hsieh *et al.*, 1994; Nixon *et al.*, 1994; Tu *et al.*, 1995; Xu *et al.*, 1996) then their proteolysis and loss would reduce the spacing between neurofilaments and lead to their compaction.

It may therefore be hypothesised, in DAI and models of TAI, that neurofilament proteolysis takes place after dephosphorylation. It has been proposed that changes in the permeability of the axolemma may lead to alteration in ionic regulation which, in turn, results in activation of phosphatase, dephosphorylation of neurofilament side-arms, followed by their proteolysis resulting in the observed compaction of neurofilaments (Maxwell *et al.*, 1997b; Povlishock *et al.*, 1997).

II.b. Changes in the number of neurofilaments

As mentioned before different responses in the number of neurofilaments occurred in the smaller and larger fibres with intramyelinic spaces (diameter > 2.00 μm) where in the former the number of neurofilaments increased in axons up to 1.00 μm in diameter while in the latter the number of neurofilaments decreased (tables 9 and 13;

pages 121 and 164) (Jafari *et al.*, 1997).

Immunocytochemical studies have demonstrated an increase in the immunolabelling for and the number of 68 kD neurofilament subunits one hour after traumatic brain injury in rats and cats. In these experiments most of these immunoreactive neurofilaments were aligned parallel to the long axis of the axon at early time points after injury (Yaghmai and Povlishock, 1992). But this parallel alignment was increasingly lost over 2-6 hours postinjury (Yaghmai and Povlishock, 1992). However, no quantitative data was given for any change in axonal calibre and therefore no correlation can be drawn concerning changes in axonal parameters with the change in the number or increased labelling of neurofilament subunits.

Increase in the number of neurofilaments in the present study cannot be through the synthesis of neurofilaments in the cell body and their transport to the site of injury, because it has been established that neurofilament proteins are delivered by the slowest subcomponent (SCa) of axonal transport (Hoffman *et al.*, 1984, 1985a; Parhad *et al.*, 1987; Monaco *et al.*, 1989). The rate of transport of neurofilament protein in the guinea pig optic nerve axons has been found to be 0.25 mm per day (Black and Lasek, 1980) and therefore it would take a much longer time than 4 hours after stretch-injury for neurofilament proteins to be delivered, over a distance of approximately 6 mm from the retinal ganglion cell, to the site of injury. It took 20 days after axotomy for neurofilament proteins to be transported to the proximal stump of regenerating fibres (see Hoffman *et al.*, 1984). Even if there was an increased rate of neurofilament transport, as was found for example after 2,5-hexanedione-induced axonal neuropathy in the rat, with a rate four times (0.80 mm/day) higher than the normal value (Monaco *et al.*, 1989), the rate of transport would still be too slow for newly polymerised

neurofilaments to reach the site of axonal injury from retinal ganglion cells in the guinea pig optic nerve 4 hours after stretch-injury. Therefore, the increase in the neurofilament number must occur locally within the injured axons. There is a critical concentration of 40 $\mu\text{g/ml}$ or 0.6 μM of unassembled NF-L molecules in a small and constant pool which remains in dynamic equilibrium with subunits within the NF-L filament (Angelides *et al.*, 1989). Soluble NF-L subunits which are kinetically active undergo subunit exchange or polymerisation under physiological salt conditions that are normally present in the axon (Angelides *et al.*, 1989). It has been suggested that these soluble subunits might be drawn from the free monomer pool and used for NF-L polymerisation during regeneration (Angelides *et al.*, 1989). However, experimental and kinetic analyses have shown that subunit dissociation is essential for subunit exchange and the rate of exchange is limited by subunit dissociation (Angelides *et al.*, 1989). The half-time for subunit exchange between phosphorylated filaments is 80 minutes while for dephosphorylated subunits it is approximately 2 minutes (Angelides *et al.*, 1989). There is therefore a strong possibility that dephosphorylation could regulate filament destabilisation, subunit dissociation and consequently the occurrence of a transient soluble pool of dephosphorylated subunits, which provide a source of precursors for neurofilament assembly. The newly exchanged subunits can then be stabilised by phosphorylation (Angelides *et al.*, 1989). In invertebrates there is good evidence that new neurofilament polymers are formed from the existing pool of cytoskeletal proteins in regenerative sprouts from squid giant axons (see Morris and Lasek 1984) after injury. It has been posited that at least some axonal neurofilament proteins may remain in an axon as monomers in their non-organelle form (Angelides *et al.*, 1989; Okabe *et al.*, 1993) and that there is a capacity for local assembly of

monomers into filaments within the axon (Okabe *et al.*, 1993). Both a continuous movement and a nonuniform distribution of deposited neurofilament proteins are present along the length of the axon, and thus exchange between soluble neurofilament proteins and the existing neurofilament lattice could take place at different sites along the length of the axon. This reservoir of soluble subunits could therefore provide an axon with the unusual ability to undergo morphological changes at any point along its length without direct participation, or requirement, for novel protein synthesis (Angelides *et al.*, 1989). It has also been found that, at least in axotomised sciatic nerve in C57BL/6/Ola and C57BL/Ola mice (Watson *et al.*, 1993; Glass and Griffin, 1991, 1994), there is also a retrograde movement of cytoskeletal proteins. Therefore it may be hypothesised that the increased number of neurofilaments in axons where neurofilament compaction occurred, especially those with axonal diameter less than 1.00 μm 4 hours after stretch-injury to the guinea pig optic nerve, and the increased content of NF-L after moderate traumatic brain injury (fluid-percussion and controlled cortical impact injury) in rats and cats (Yaghai and Povlishock, 1992), result from a local NF-L polymerisation or assembly with subunit exchange (*vide supra*) from the local pool of unassembled neurofilament protein monomers (Angelides *et al.*, 1989; Okabe *et al.*, 1993). It may also be hypothesised that subunit exchange may occur in damaged NF-L to allow repair or regeneration.

The significant loss of axonal neurofilaments in fibres with an axonal diameter greater than 2.00 μm (table 9) (Jafari *et al.*, 1997) at 4 hours after injury in the present study through Wallerian degeneration is already discussed in pages 191-192.

Thus it may be hypothesised in the present study that preservation of neurofilaments or even an increase in their number in smaller axons (diameter < 1.00

µm) and a loss of their number in larger axons (diameter > 2.00 µm) could result from either differential sensitivities of axonal calpains to intracellular levels of calcium concentration as documented in normal and Ola mice (Glass *et al.*, 1994) or hypothetical differential distribution of calpains in the axon (Hamakubo *et al.*, 1986). The present study provides the first quantitative, morphological evidence for different pathological responses in respect of changes in the number of neurofilaments between smaller axons (diameter < 1.50 µm) and the larger axons (diameter > 2.00 µm) and between larger fibres with intramyelinic spaces and fibres with periaxonal space(s) 4 hours after stretch-injury in the guinea pig optic nerve.

II.c. Changes in the number of Microtubules

There is a dynamic equilibrium between a monomeric and polymeric state of tubulins which can be shifted either to polymerisation to form microtubules, or depolymerisation to form monomers. Several studies have shown the existence of cold-stable and cold-labile microtubules in the axonal extract (Webb and Wilson, 1980; Brady *et al.*, 1984; Watson *et al.*, 1990).

Depolymerisation of cold-labile microtubules in mammalian brain tissue has been reported to take place quickly under even a low concentration of calcium (Weisenberg, 1972; Gaskin *et al.*, 1975; Olmsted and Borisy, 1975; Nishida and Sakai, 1977). Cold-stable microtubules in the crude extract of rat brain were almost completely disassembled in the presence of 400 µM free calcium but purified cold-stable microtubules were quite insensitive to free calcium with a concentration of less than 5 mM and did not respond to even a high concentration of calmodulin in the absence of calcium (Job *et al.*, 1981). Addition of 0.5 mM calcium ions alone to microtubules

isolated from bovine brain induced a slow but continuous disassembly of the microtubules. However, when both 0.5 mM calcium ions and μM or mM calpain were added there was a rapid disassembly of microtubules which was completed within 5 minutes (Billger *et al.*, 1988). In the present study it may be hypothesised that activation of μM calpain could occur in the larger fibres with periaxonal space(s) where there is a compaction of neurofilaments. It also could happen in the larger fibres with intramyelinic spaces where there is a significant loss of both microtubules and neurofilaments (table 9; pages 121 and 137). But, since there is a significant loss of neurofilaments in these fibres, therefore, as mentioned above, for the complete loss of NF-L and NF-M a concentration of at least 0.20 mM Ca^{2+} , is needed (Glass *et al.*, 1994, reviewed by Maxwell *et al.*, 1997b) and under these conditions the disassembly of microtubules would occur (Billger *et al.*, 1988). But in the present study all of microtubules were not lost, some were preserved both in the larger fibres with periaxonal space(s) and those with intramyelinic spaces. One reason for this could be that cold-labile and cold-stable microtubules differ in their sensitivities to calcium (Nishida and Sakai, 1997). Thus it may be hypothesised that the more sensitive, cold-labile, microtubules are more vulnerable to disassembly than the more stable, cold-stable, microtubules which require a calcium concentration of greater than 5 mM for disassembly (Job *et al.*, 1981).

III. Placement of my results in the context of the literature and the potential for the development of post-traumatic therapies

The present work provides the first, quantitative data that demonstrates that (i) different sized axons respond in different ways to stretch-injury and (ii) that microtubules respond in a different way to neurofilaments.

The results demonstrate that marked changes occur in the axonal cytoskeleton by 4 hours after injury. The data that I have generated provides a widely based analysis of these changes. Since I have generated, for the first time, such quantitative, morphometric data the scene is now set to allow analysis of the influence of therapeutic interventions upon axonal cytoskeletal responses to non-disruptive axonal injury. The current literature indicates a disparity between long term outcome after traumatic brain injury as measured through behavioural tests and structural changes in the injured brain (Marion and White, 1996; Dixon *et al.*, 1998).

But the criteria for assessment of levels or degrees of, for example, axonal injury were probably not appropriate since Marion and White (1996) used the number of immunohistochemically labelled axonal bulbs as an indicator of axonal injury. But, before axons form axonal bulbs they demonstrate a number of pathological changes, the most important being changes in axonal calibre and the formation of axonal swellings (Maxwell *et al.*, 1997). These changes in axons importantly occur before secondary axotomy. Degeneration bulbs occur only after secondary axotomy. The present study is important in that the included data provides a detailed description of changes occurring in injured axons before secondary axotomy. If therapeutic interventions are to be developed in the near future it will be essential to be able to ascertain, using quantitative morphometric parameters, what influence those therapies

have upon the structure of the axonal cytoskeleton such that preservation of axonal integrity can be demonstrated using unbiased, quantitative techniques. The data included in the present thesis provides just such essential information. It is also important that we now have good, quantitative data concerning changes in the axon prior to axotomy. Only comparison of cytoskeletal responses in injured axons in animals to which post-traumatic therapy has been applied will allow the unequivocal demonstration of the effectiveness of the aforementioned therapies.

CONCLUSIONS

The following conclusions were obtained.

At 4 hours after stretch-injury to the right optic nerve of the adult guinea pig: -

1. The most severe axonal damage occurs in the middle segment of the stretch-injured optic nerve.
2. The number of axons in the smallest bin size (diameter $< 0.50\ \mu\text{m}$) increased in all three segments of the optic nerve.
3. There was a decreased number of axons within the bin size of $0.51\text{-}1.00\ \mu\text{m}$ axonal diameter in the second segment.

In the second segment of injured nerves:

4. Compaction of neurofilaments occurred in axons with a diameter less than $1.50\ \mu\text{m}$.
5. The degree of reduction in neurofilament spacing differed between axons of different size: to 53%, 41% and 35% of control values in axons of $0.00\text{-}0.5$, $0.51\text{-}1.0$ and $1.01\text{-}1.5\ \mu\text{m}$ diameter respectively. Compaction of neurofilaments did not occur in axons with a diameter between $1.50\ \mu\text{m}$ and $2.00\ \mu\text{m}$.
6. Compaction of neurofilaments occurred over tens of microns along the length of axons of $1.5\ \mu\text{m}$ or less diameter. In the smallest axons (diameter $< 0.50\ \mu\text{m}$) neurofilaments were compacted to form a central filamentous core.
7. The number of neurofilaments was increased in axons with a diameter of less than $1.00\ \mu\text{m}$.
8. Compaction of neurofilaments in the smallest axons (diameter $< 0.50\ \mu\text{m}$) was as a result of both an increase in the number of neurofilaments and a reduced spacing between them. But, compaction of neurofilaments in fibres with an axonal diameter

of between 1.00- 1.50 μm was due to a reduced spacing between neurofilaments with no change in their number.

9. Neurofilament compaction occurred in larger fibres with a periaxonal space(s).
10. In the majority of larger fibres (axonal diameter > 2.00 μm) neurofilament compaction did not occur. Rather there was an increased spacing between filaments. This is referred to as “dispersion” of neurofilaments.
11. In the majority of the larger fibres (axonal diameter > 2.00 μm) the number of neurofilaments did not increase, rather there was a significant decrease in their number. The increase in the spacing between neurofilaments was therefore correlated with the loss in their number in the axoplasm.
12. In larger fibres with periaxonal space(s) there was no significant increase in the number of neurofilaments. Compaction of neurofilaments in these fibres was therefore due to the decrease in spacing between neurofilaments.
13. Compaction of neurofilaments occurred at different foci along the length of some larger nerve fibres. Compaction of neurofilaments at these foci was associated with an expansion of fibre calibre to form a so-called “balloon-like” appearance. The axoplasm of these nerve fibre between these foci had a normal appearance.
14. Compacted neurofilaments were aligned parallel to each other in longitudinal sections. There was a low number of microtubules among these compacted neurofilaments.
15. At some foci after stretch-injury, compaction of neurofilaments was associated with axolemmal discontinuity and the loss of its close relationship with the axonal cytoskeleton and the myelin sheath. Some fragments of the axolemma were extended into the periaxonal space(s). There was disruption of the axolemma such

that large holes were present. In these foci the myelin sheath was grossly disrupted and expanded to form a “balloon-like” appearance. These foci probably represent the most severe form of axonal damage.

16. At a mild degree of axonal damage, there was just a misalignment of neurofilaments and microtubules.

17. The G-ratio, that is the ratio of axon diameter to fibre diameter, was reduced significantly only in the smallest fibres (axonal diameter $< 0.50\ \mu\text{m}$) and the larger fibres with periaxonal space(s) where in both fibres there was a shrinkage of axonal calibre.

18. Microtubule spacing did not increase significantly in fibres with an axonal diameter less than $1.50\ \mu\text{m}$. But there was a significant increase in microtubule spacing in larger fibres with an axonal diameter greater than $1.50\ \mu\text{m}$. There was a significant increase in microtubule spacing in fibres with a periaxonal space(s).

19. A significant decrease in the number of microtubules occurred in both larger fibres (diameter $> 2.00\ \mu\text{m}$) even in those larger fibres with periaxonal space(s), where there was significant compaction of neurofilaments. The increase in spacing between microtubules in these fibres was correlated with a loss of their number.

The above findings provided strong evidence that differential changes occurred in axons and fibres of different sizes and supported the hypothesis that there is a spectrum of pathological changes in different sizes of axons/fibres after stretch-injury to the optic nerves of the guinea pig.

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